Analysis of Genetic Diversity and Evolution through Recombination of Beak and Feather Disease Virus

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A thesis submitted in partial fulfilment of the requirements for the

Degree of Master of Science in Microbiology

at the University of Canterbury

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2012

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Acknowledgements

The completion of this thesis required first the collection of blood and feather samples from different countries, which was not possible for me to do. As such I would like to thank the people involved in collecting such samples. From New Zealand, Jason van de Wetering, Maddie van de Wetering, and Moira Pryde of the Department of Conservation (DOC) for collecting the South Island samples, and Kate McInnes, also from DOC, for coordinating the entire study and allowing me to be a part of it. From New Caledonia, Almudena Lorenzo, Jean-Paul Chenuet, Marianne Bonzon, Celine Marchal, and Laurent Vignon, with the additional assistance of Bethany Jackson and Arvind Varsani from New Zealand. From Poland, Drs Tomasz Piaseki and Klaudia Chrzastek, who not only collected all the samples, but pre-processed them before sending them to New Zealand. Without the efforts of all of these people, there would have been no thesis.

I would also like to acknowledge my supervisor, Dr Arvind Varsani, for his help, support, patience, and guidance, and most importantly, well-timed provision of chocolate whenever things started getting too stressful! It has truly been a pleasure to work under his supervision, and I would do it all again in a heartbeat.

I am also grateful for the help, support and advice my fellow lab-mates have provided during the last year and a half. I had the privilege of working with a fantastic group of people, all of whom were very supportive. In particular, the impromptu coffee and cake breaks on those days when things just didn't go right were much appreciated! Cheers, you guys.

My husband, Matt Roche, has supported me throughout this six year journey, although it was originally supposed to be only four years! My daughter Taylah has also had to put up with her mum being busy studying for the last six years, and I thank them both for their patience and understanding.

Abstract

Beak and feather disease virus (BFDV), a non-enveloped, icosahedral virus with a circular single stranded DNA (ssDNA) genome, is the causative agent behind psittacine beak and feather disease (PBFD), an often fatal disease affecting parrots. Symptoms include feathering abnormalities, loss of feathers, and occasionally beak and claw deformities. BFDV-induced immunosuppression results in an increased susceptibility to secondary microbial infections, which is often the cause of death in infected parrots. There is no cure, no effective treatment, and no protective vaccine for BFDV. The international trade in exotic parrots has facilitated the spread of BFDV, so that it now has a global presence. Given that over a quarter of the currently recognised 356 psittacine species are considered to be at risk of extinction in the wild, the worldwide presence of BFDV, coupled with its extreme environmental stability, poses serious concerns for the future of some of the worlds most endangered parrots.

That genetic diversity exists among BFDV isolates has been established, yet in the 14 years since the genome was fully sequenced, very few full-length BFDV genome sequences have been deposited in GenBank, despite the technology to rapidly isolate and amplify entire circular ssDNA genomes being readily available. Most studies have sequenced just a portion of the genome, usually one of the open reading frames (ORFs) encoding the major viral proteins, to investigate phylogenetic relationships between isolates. However the two major BFDV ORFs, encoding the replication associated protein (Rep) and the capsid protein (CP), have been shown to evolve at different rates, with the functional Rep being generally more conserved while CP is more variable. When also considering the fact that ssDNA viruses are notoriously recombinant, it becomes clear that an analysis based on a portion of the genome is unlikely to accurately establish evolutionary relationships. Therefore the focus of the studies described in this thesis was on isolation and amplification of full-length BFDV genomes from avian blood and feather samples that first tested positive to a PCR-based BFDV screening method. Samples were collected by appropriately trained people in New Zealand, New Caledonia, and Poland, before being sent to the University of Canterbury for molecular and bioinformatic analysis. The sequences of the BFDV genomes from each region were compared to each other and to all other full BFDV genome sequences publically available in GenBank, to compare the genetic diversity among these isolates. Recombination analyses were also performed, to assess how recombination is impacting on the evolution of BFDV.

New strains of BFDV and new subtypes of existing BFDV strains were discovered, indicating that the global genetic diversity may be greater than previously thought. Many strains also proved to be recombinants, in particular those from Poland. Europe has had a long history with importing and breeding exotic parrots, and the high degree of recombination among the Polish BFDV isolates coupled with the number of previously unsampled strains is an example of how maintaining populations of multiple species in captivity enables evolution through recombination, and emergence of novel viral strains.

Full genome analyses can also enable tracking the source of an infection. A total of 78 full genome sequences from 487 samples tested were deposited into GenBank as a direct result of the work undertaken as part of this thesis, thereby adding to the existing knowledge base regarding BFDV. With continued global sampling and full genome analysis it may one day be possible to trace the history of BFDV to its original emergence.

Chapter one: Introduction

1.1 The Family *Circoviridae*

The family *Circoviridae* currently represents the smallest known autonomously replicating animal viruses, divided into two genera: *Gyrovirus,* which currently has only one recognised member, Chicken anaemia virus (CAV); and *Circovirus,* which currently recognises 11 species that infect birds and pigs (Biagini *et al.*, 2012). The *Circoviridae* family is classified as belonging to Group II under the Baltimore system, the members of which have a single-stranded DNA (ssDNA) genome, while the prefix 'circo' (or 'gyro') indicates that the ssDNA genome is arranged in a closed circular conformation. Virions of the members of this family display icosahedral symmetry, are non-enveloped and have an average diameter of ~20-25nm (Crowther *et al.*, 2003; Khayat *et al.*, 2011; Paré and Robert, 2007; Todd, 2000). The genome size among members of the *Circoviridae* family ranges from about 1.7 to about 2.3kb, but genome organisation differs between the circoviruses and the gyroviruses (Biagini *et al.*, 2012).

All viruses rely on host cell machinery for replication, but the *Circoviridae*, with their small genomes encoding few proteins, are particularly dependent on actively dividing host cells for their own replication. Once inside the nucleus of the host cell, the ssDNA genome is used as a template for the formation of a complementary strand of DNA, making a double-stranded DNA (dsDNA) intermediate, also known as the replicative form (RF). The double-stranded replicative form is then used as a template for the production of mRNA, with open reading frames (ORFs) potentially on both the original virion strand (V) and also on the complementary strand (C) of the replicative intermediate (Biagini *et al.*, 2012; Ilyina and Koonin, 1992; Mankertz *et al.*, 2004; Mankertz *et al.*, 1997).

Circoviridae family members have been reported to be very environmentally stable and highly resistant to many of the methods commonly employed to inactivate viruses, such as high temperature, low pH, organic solvents and commercial disinfectants, making environmental contamination an important route of transmission, and presenting difficulties in control of disease outbreaks (Urlings *et al.*, 1993; Welch *et al.*, 2006; Yuasa, 1992; Yuasa *et al.*, 1979).

1.2 Genus *Gyrovirus*

Currently the sole recognised member of the *Gyrovirus* genus, *Chicken anaemia virus* (CAV) is distinguishable from members of the *Circovirus* genus both by its size and its genome organisation. It is the largest known member of the *Circoviridae* family, with virions of approximately 25nm in diameter, and genomes of about 2.3kb with a negative sense organisation (Rosenberger and Cloud, 1998; Schat, 2009). CAV has stood alone in the genus since its discovery in Japan in 1979 (Yuasa *et al.*, 1979), but the recent discovery of viruses with CAV-like genomes from chickens (dos Santos *et al.*, 2011; Rijsewijk *et al.*, 2011), human skin swabs (Sauvage *et al.*, 2011), and blood (Maggi *et al.*, 2012), and from human faecal matter and chicken meat (Chu *et al.*, 2012; Phan *et al.*, 2012), indicates that there may be significantly more diversity within this genus.

1.2.1 Genome organisation and replication

The negative sense genome of CAV has three partially overlapping open reading frames (ORFs) coding for three viral proteins,VP1, VP2, and VP3 (Noteborn *et al.*, 1991) (Fig. 1.1). All three are found on the complementary strand, or positive sense strand, of the RF (Todd, 2000). The largest protein of the three, VP1, is a 52kDa capsid protein; VP2 is a 24kDa protein phosphatase; and VP3, the smallest protein at 13kDa, plays a role in apoptosis, thus has been called apoptin (Chandratilleke *et al.*, 1991; Noteborn *et al.*, 1994a; Noteborn, 2004; Peters *et al.*, 2002). The three proteins are translated from a single 2.1kb polycistronic mRNA through use of alternate start codons (Noteborn *et al.*, 1992), but there is evidence this mRNA may also be spliced to produce alternative transcripts, although as yet it is unknown what the products of these spliced mRNAs do, or even if they are translated (Kamada *et al.*, 2006). The 5' untranslated region (UTR), located between the polyadenylation site and the transcription start site of the polycistronic mRNA, contains the only region with promoter/enhancer activity, which controls transcription and replication of the virus (Noteborn *et al.*, 1992; Noteborn *et al.*, 1994b). It is thought that CAV replicates via the rolling circle replication (RCR) process common to other circular ssDNA viruses. However, although the genome of CAV contains a similar nonanucleotide motif associated with initiation of RCR in the UTR, this is not located at the top of a potential stem-loop structure as it is with other circular ssDNA viruses (Bassami *et al.*, 1998). A putative hairpin structure in the CAV genome is located approximately four nucleotides after the stop codon for the mRNA, and the nonanucleotide sequence is approximately 100bp downstream from the hairpin (Claessens *et al.*, 1991; Noteborn *et al.*, 1991). Also CAV does not appear to encode a replication-associated protein (Rep) as other genomes employing RCR do, but three amino acid sequence motifs usually present in the Rep and associated with RCR (Ilyina and

Koonin, 1992) (Table 1.1) are found on VP1 (Niagro *et al.*, 1998), indicating that in the case of CAV, the capsid protein may be associated with DNA replication.

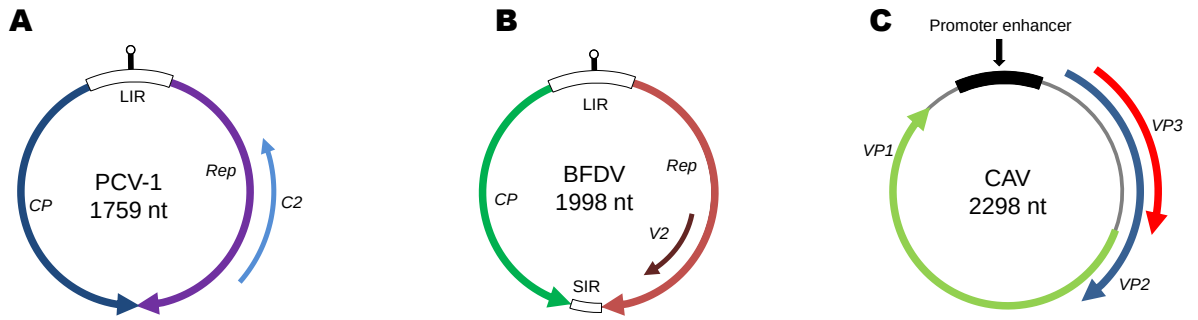


Fig. 1.1. Cartoon representation of the genome organisation of (A) *Porcine circovirus-1*, (B) *Beak and feather disease virus*, and (C) *Chicken anaemia virus*, showing the locations of the major ORF's. LIR = long intergenic region, SIR = short intergenic region.

1.2.2 Virion morphology

Virions are round, have no envelope, and display icosahedral symmetry. Negative stained particle electron micrographs comparing CAV to circoviruses show that CAV is approximately 30% larger than the circoviruses, and that it has a distinctive surface morphology, giving it a rough appearance that the circoviruses do not have (Todd *et al.*, 1991). Crowther *et al.* (2003) determined the three-dimensional structure from electron micrographs showing that the virion of CAV is made up of 60 identical subunits, formed from the VP1 capsid protein, with groups of five subunits forming a pentagonal trumpet-shaped capsomere, giving the rough appearance which also helps to distinguish it from the circoviruses, which have a much smoother appearance when viewed under the electron microscope.

1.2.3 Pathology of Chicken anaemia virus

CAV is known to infect chickens in the commercial meat and egg industries worldwide (Rosenberger and Cloud, 1998). Transmission can be both vertical, from hen to chick, and horizontal, most likely through the faecal-oral route (Miller and Schat, 2004). Horizontal transmission is enhanced by the fact that CAV is extremely resistant to inactivation. While infection is common, development of clinical disease symptoms is related to the age of the bird at the time of infection. Exposure to CAV in three week old hatchlings appears to result in a subclinical infection, and maternal antibodies provide protection to chicks up until about three weeks of age, after which time horizontal transmission results in subclinical infection. Vertical transmission occurs when a CAV-naïve hen

becomes infected during egg production, although the hen itself does not show clinical signs of infection (Vielitz and Landgraf, 1988). Offspring vertically infected are viable and hatch normally, but suffer from increased mortality and develop disease symptoms within the first two weeks of life (McNulty, 1991; Todd, 2000). Symptoms include depression and lethargy, with anaemia, skin lesions, subcutaneous or intramuscular haemorrhage, and atrophy of the thymus, bursa of Fabricius, and spleen also being observed (McNulty, 1991; Miller and Schat, 2004; Rosenberger and Cloud, 1998; Todd, 2000). The virus targets haemocytoblasts in the bone marrow and T lymphocyte precursor cells in the thymus (Adair, 2000). Haemocytoblasts are the progenitor cell for erythrocytes (red blood cells, destruction of which leads to anaemia), thrombocytes (involved in blood clotting, destruction of which leads to haemorrhage), and heterophils (similar to mammalian neutrophils, a white blood cell involved in the immune response). Destruction of these cells is thought to be by apoptosis caused by VP3, or apoptin, although how this occurs is still unclear (Jeurissen *et al.*, 1992). The loss of heterophils and T lymphocytes, which play a role in cell-mediated immunity, effectively reduces the efficiency of the immune system, leaving the infected chicks susceptible to secondary infections (Adair, 2000; Schat, 2009). However, provided they do not succumb to secondary infection, young chicks can recover from CAV infection as the immune system matures and B cells unaffected by CAV can begin to produce CAV antibodies (Adair, 2000).

1.3 Genus *Circovirus*

The type species for the genus *Circovirus* is *Porcine circovirus*-1 (PCV1), but all members share similar morphology and genome organisation. Circoviruses exhibit strong host specificity, with two of the 11 currently recognised species infecting pigs (PCV1 and PCV2) and the remainder infecting birds (*Beak and feather disease virus*, BFDV; *Canary circovirus*, CaCV; *Duck circovirus*, DuCV; *Finch circovirus*, FiCV; *Goose circovirus*, GoCV; *Gull circovirus*, GuCV; *Pigeon circovirus*, PiCV; *Starling circovirus*, StCV; and *Swan circovirus*, SwCV) (Biagini *et al.*, 2012). Tentatively included, but yet to be accepted by the International Committee on Taxonomy of Viruses (ICTV), is a Raven circovirus (Biagini *et al.*, 2012; Stewart *et al.*, 2006). However the recent isolation of circoviruses from dogs (Kapoor *et al.*, 2012), fish (Lőrincz *et al.*, 2011; Lőrincz *et al.*, 2012), bat guano (Li *et al.*, 2010b), and from human and primate faecal matter (Blinkova *et al.*, 2010), indicates a greater diversity and host range than previously thought (Fig. 1.2). The best characterised of the circoviruses are PCV1, PCV2 (together hereafter referred to simply as PCV), and BFDV, which affects birds in the order Psittaciformes, encompassing the parrot and cockatoo families, and as such, further general discussion of circoviruses shall focus on these species.

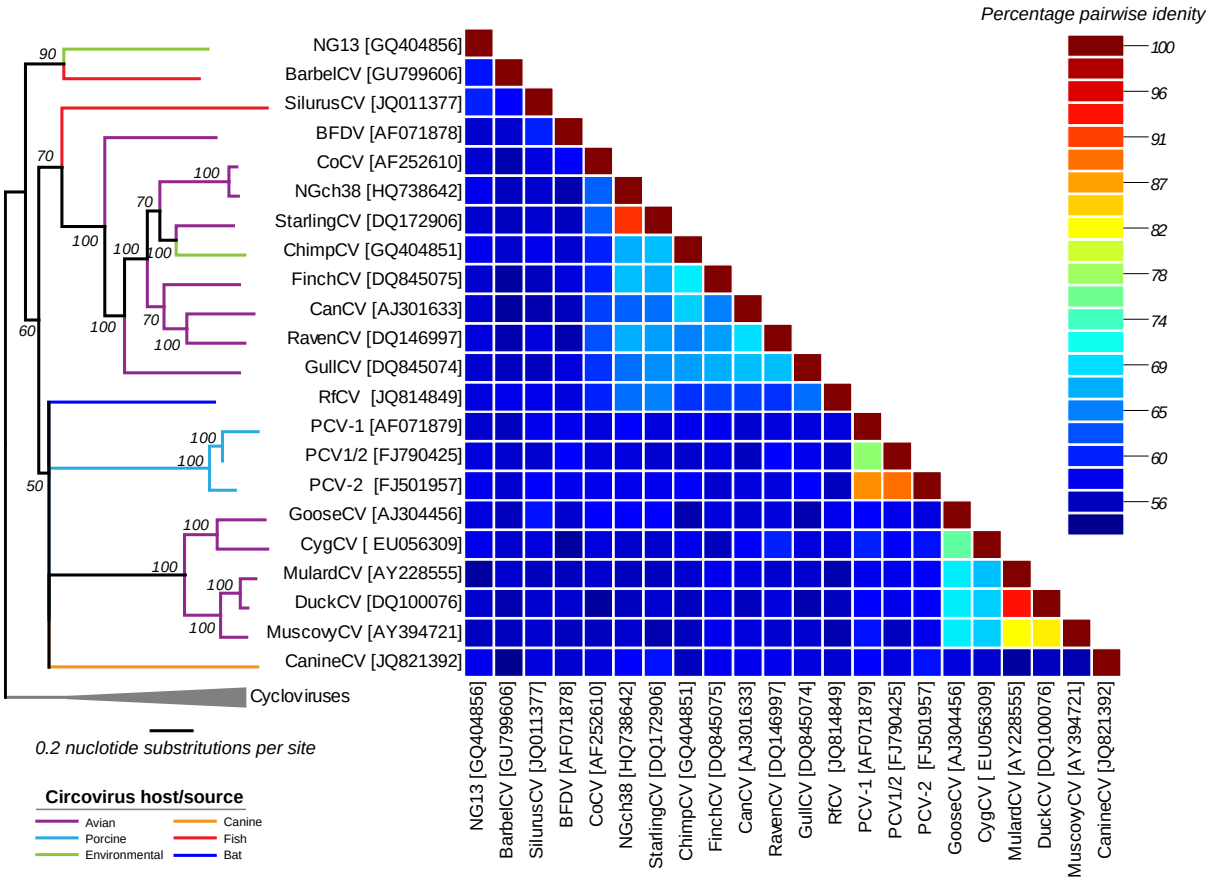


Fig. 1.2. Maximum-likelihood phylogenetic tree constructed in PHYML using model GTR+I+G4 (left) and two dimensional representation of pairwise (pairwise deletion of gaps) nucleotide sequence identity (right) between avian circoviruses, porcine circoviruses, and other circoviruses recently isolated.

1.3.1 Genome organisation and replication

The genomes of circoviruses range in size from ~1.7kb (PCV), the smallest among the *Circoviridae*, to the ~2kb genome of BFDV (Biagini *et al.*, 2012). All circovirus genomes are composed of closed circular ssDNA, and have an ambisense organisation, with ORFs located both on the encapsidated virion strand and the complementary strand of the dsDNA RF (Todd, 2000). Two major ORFs are present in all circoviruses, one on the virion strand (V1) and one on the complementary strand (C1), coding for the 35.7kDa replication-associated protein (Rep) and the 27.5kDa capsid protein (CP) respectively (Niagro *et al.*, 1998) (Fig. 1.1). The Rep and CP of PCV and BFDV are homologous, and the circovirus Reps also share some similarity with Reps of other circular ssDNA plant-infecting viruses from the *Geminiviridae* family and the *Nanoviridae* family, leading to the suggestion that circoviruses represent an evolutionary intermediate between the two families (Niagro *et al.*, 1998). It has also been suggested that the Rep protein in circoviruses is a recombinant, with the N-terminal portion showing close similarity to the N-terminal region of the Rep from nanoviruses, and the C-

terminal portion being related to an RNA-binding protein from a single-stranded RNA picorna-like virus, probably a calicivirus (Gibbs and Weiller, 1999). One point of difference between PCV and the rest of the Rep-encoding viruses is that two Reps are transcribed in PCV, the full length Rep and a shorter, spliced Rep (Rep'), both of which are needed for DNA replication (Mankertz and Hillenbrand, 2001). The Reps in circoviruses contain four amino acid motifs conserved among circular ssDNA genomes employing the rolling circle replication (RCR) method of DNA replication (Ilyina and Koonin, 1992), indicating that circoviruses also replicate via this method (Table 1.1). Interestingly, while Rep of PCV contains all four motifs, Rep' contains only three, lacking the P-loop, a putative helicase domain with divalent ion-dependant ATPase activity (Cheung, 2011; Ilyina and Koonin, 1992; Steinfeldt *et al.*, 2007). The intergenic region (IR) between the 5' ends of the two major ORFs contains a potential stem-loop structure that has at its apex the origin of replication, a nonanucleotide sequence NANTATTAC (where N is a variable nucleotide) that is highly conserved among circoviruses, geminiviruses and nanoviruses, with the circovirus consensus sequence being TAGTATTAC (Rosario *et al.*, 2012b). In PCV, located close to the right of the stem-loop structure are four hexameric repeats, 5'-CGGCAG, designated H1 to H4, with a five bp gap between H2 and H3 (Steinfeldt *et al.*, 2001). The Rep/Rep' complex binds to the right side of the stem-loop structure and to H1/H2, from which position it is able to nick the nonanucleotide sequence on the virion strand between nucleotides seven and eight (NANTATT/AC), leaving a free 3'-OH to act as a primer for the host cell DNA polymerase to extend, forming a new strand (Mankertz *et al.*, 2004; Steinfeldt *et al.*, 2006). The BFDV genome has two octanucleotide repeats located directly after the stem-loop structure, 5'-GGGCACCG (Bassami *et al.*, 1998), which probably act as a binding site for the BFDV Rep protein.

Although many more ORFs that potentially encode proteins have been identified in both PCV and BFDV (Bassami *et al.*, 1998; Bassami *et al.*, 2001; Hamel *et al.*, 1998; Meehan *et al.*, 1997; Meehan *et al.*, 1998; Morozov *et al.*, 1998), the function of only one other ORF is known. ORF3, or C2, the third largest ORF in the PCV genome, was shown to encode a protein that was not essential for viral replication, but played a role in apoptosis (Chaiyakul *et al.*, 2010; Liu *et al.*, 2005), similar to VP3 in CAV.

1.3.2 Virion morphology

As with CAV, three dimensional reconstruction from electron micrographs show that both PCV and BFDV have virions consisting of 60 identical subunits, formed from the C1 capsid protein (Crowther *et al.*, 2003). While the icosahedral virions have the same general structure as CAV, with groups of five subunits forming a pentagonal capsomere, these lie flatter than the trumpet-shaped

capsomeres of CAV, giving the virions a smoother appearance under the electron microscope (Crowther *et al.*, 2003; Todd *et al.*, 1991). Virions of BFDV and PCV range from 1.7kb to 2.3kb, with an average diameter of 20.5nm (Crowther *et al.*, 2003).

Table 1.1. Table of the four conserved amino acid motifs observed in Rep of all circular ssDNA viruses employing the rolling circle replication (RCR) method of DNA replication, shown here pertaining to selected circoviruses.

				P-loop (Motif 4)	
	Motif 1	**Motif 2**	**Motif 3**	**Walker A**	**Walker B**
NG13	FTLNN	HLQGFF	YCSKSGNI	GPPGCGKS	VIIDDF
Barbel CV	FTLNN	HLQGFL	YCTKGGDT	GDPGCGKS	VIVDDF
Silurus CV	FTINN	HLQGFL	YCSKETTY	GPPGSGKS	VVMDDF
BFDV	FTLNN	HLQGYF	YCSKEGDV	GPPGCGKS	VILDDF
CoCV	FTLNN	HLQGFV	YCSKEGNV	GPPGCGKS	VIIDDF
NGch38	FTLNN	HLQGFI	YCSKEGNV	GPPGCGKS	VVIDDF
StarlingCV	FTLNN	HLQGFI	YCSKEGDV	GPSGVGKS	VIIDDF
ChimpCV	FTLNN	HLQGYL	YCSKEGDV	GPSGVGKS	VVIDDF
FinchCV	FTLNN	HLQGFL	YCGKEGDV	GPSGCGKS	VIIDDF
CanCV	FTLNN	HLQGFL	YCSKENDV	GPSGVGKS	VIMDDF
RavenCV	FTLNN	HLQGYI	YCTKDGDV	GPSGVGKS	VVIDDF
GullCV	FTLNN	HLQGFM	YCGKDGEI	GPPGCGKS	VIIDDF
RfCV	FTINN	HLQGFI	YCTKEETY	GEPGSGKS	VVLDDF
PCV-1	FTLNN	HLQGFA	YCSKEGHI	GPPGCGKS	VVLDDF
PCV1/2	FTLNN	HLQGFA	YCSKEGHI	GPPGCGKS	VVLDDF
PCV-2	FTLNN	HLQGFA	YCSKEGNL	GPPGCGKS	VVIDDF
GooseCV	FTINN	HLQGFL	YCSKESTY	GRPGSGKS	VVMDDF
CygCV	FTLNN	HLQGFL	YCAKESTY	GPPGTGKS	VVMDDF
MulardCV	FTINN	HLQGFL	YCAKESTY	GPPCTGKS	VVMDDF
DuckCV	FTINN	HLQGFL	YCSKESTY	GPPGTGKS	VVMDDF
MuscovyCV	FTLNN	HLQGFA	YCSKEGNL	GPPGCGKS	VVIDDF
CanineCV	FTINN	HLQGFV	YCSKGGDL	GPPGCGKS	VILDDF

1.3.3 Pathology of Circoviruses

The majority of circovirus infections do not appear to cause obvious symptoms, however PCV2 and BFDV have been established as the causative agents behind well documented and studied diseases (Hamel *et al.*, 1998; Morozov *et al.*, 1998; Ritchie *et al.*, 1989b). Unlike PCV1, which is generally regarded as non-pathogenic, PCV2 has been implicated in a number of porcine diseases (Grau-Roma

et al., 2011), most notably post weaning multisystemic wasting syndrome (PWMS), and BFDV causes psittacine beak and feather disease (PBFD) in parrots.

1.3.3.1 PCV2 and post weaning multisystemic wasting syndrome (PMWS)

Instances of PMWS first came to light in Canada in the 1990's (Harding *et al.*, 1998), and have since been reported in most pig-farming countries worldwide (Allan and Ellis, 2000). Younger pigs, between the ages of five and 12 weeks, are most commonly affected, in some cases fatally (Allan and Ellis, 2000). Symptoms include wasting, respiratory difficulties such as dyspnoea, enlarged lymph nodes, and occasionally diarrhoea, paleness or jaundice (Allan and Ellis, 2000; Harding *et al.*, 1998). Microscopically, lesions are also present in multiple body tissues, particularly in lymphoid organs (Harding *et al.*, 1998). Virus shedding occurs via a number of routes, including nasal, oral, and faecal secretions, which have been shown to be infective to PCV2-naïve animals (Patterson *et al.*, 2011a; Patterson *et al.*, 2011b; Segalés *et al.*, 2005). Transmission occurs both horizontally and vertically, with the oronasal route the most likely for horizontal transmission (Grau-Roma *et al.*, 2011). Infection of pregnant sows with PCV2 has been associated with reproductive failure, and virus could be detected in both stillborn and liveborn piglets, indicating the ability of the virus to cross the placenta (Park *et al.*, 2005).

Infection with PCV2 does not always result in PMWS, and exactly why some pigs but not others develop clinical disease symptoms is unclear, but it has been suggested that other factors are necessary. Experimental infection with PCV2 on its own caused mild lesions similar to those seen in PMWS, but co-infection with porcine parvovirus (PPV) caused full clinical disease symptoms along with severe lesions, as seen in PMWS. Infection with PPV on its own produced no clinical symptoms, and no lesions. This would tend to suggest that PMWS is a multifactorial syndrome, with PCV2 being necessary, but on its own not necessarily sufficient to cause disease. (Allan *et al.*, 1999; Krakowka *et al.*, 2000).

1.3.3.2 BFDV and psittacine beak and feather disease (PBFD)

When first described, PBFD was thought to be confined to Old World and South Pacific psittacine species, but the discovery of characteristic lesions in New World species, and the subsequent isolation of BFDV from these birds demonstrated their susceptibility and determined it as a causative agent of PBFD (Huff *et al.*, 1988; Ritchie *et al.*, 1990; Ritchie *et al.*, 1989a). Today, BFDV has a global distribution in both wild and captive populations, in most part due to the international pet trade in exotic psittacines, and with over 60 species of psittacine birds known to be susceptible to infection with BFDV, all psittacine species are considered susceptible (Todd, 2004). PBFD can present as a

peracute, acute or chronic disease. Younger birds, neonates to fledglings, tend to suffer from the peracute and acute forms, in which sudden death occurs with no (peracute) or mild (acute) feather dystrophy (Doneley, 2003; Ritchie *et al.*, 1989a). The chronic form is more prevalent in older birds, and symptoms begin with depression, lethargy, and diarrhoea, before the feathers are noticeably affected. Typical signs of BFDV infection in feathers are retained feather sheaths, constricted or necrotic feather shafts, curled or clubbed feathers, and haemorrhage into the feather shaft (Huff *et al.*, 1988; McOrist *et al.*, 1984; Pass and Perry, 1984; Raidal, 1995; Ritchie *et al.*, 1990). Feather abnormalities are often first noticed following a moult, when the newly growing replacement feathers appear dystrophic and necrotic, and stop growing shortly after emerging from the follicle. This is progressive, and can continue through successive moults in a reasonably bilateral symmetrical fashion, until the bird is more or less bald, perhaps with patches of short, stumpy feathers remaining (McOrist *et al.*, 1984; Paré and Robert, 2007; Pass and Perry, 1984; Ritchie *et al.*, 1989a; Ritchie *et al.*, 1989b). In severe cases there may also be beak deformities, and occasionally claw deformities (Pass and Perry, 1984). This may be species specific however, as beak and claw involvement appears to be more prevalent in cockatoos than in other species (McOrist *et al.*, 1984; Ritchie *et al.*, 1989b). Possible beak deformities include elongation, transverse or longitudinal cracking, and palatine necrosis (McOrist *et al.*, 1984; Pass and Perry, 1984; Ritchie *et al.*, 1989a; Ritchie *et al.*, 1989b).

Microscopically, basophilic intracytoplasmic and intranuclear inclusions are observed in feather and feather follicle epithelium (McOrist *et al.*, 1984; Pass and Perry, 1984). Intranuclear and intracytoplasmic inclusion bodies can be found in feather and follicular epithelial cells, while macrophages present in the feather epithelium, follicular epithelium or pulp cavity can contain multiple intracytoplasmic inclusion bodies (Latimer *et al.*, 1991b; Ramis *et al.*, 1994; Sanada *et al.*, 1999). These inclusion bodies have been shown to contain BFDV viral antigen (Latimer *et al.*, 1991b; Ramis *et al.*, 1994). As well as inclusion bodies, necrosis has been observed in feather epithelial cells and occasionally the pulp cavity, along with necrosis of the basal epidermis in the beak (Latimer *et al.*, 1991b; McOrist *et al.*, 1984; Pass and Perry, 1984). This targeting of feather, follicular, and beak epithelial cells by BFDV and the resulting necrosis explains the range of feather and beak abnormalities associated with BFDV.

Inclusion bodies or viral nucleic acid have also been reported in the thymus, bursa of Fabricius, and in other organs and tissues, often associated with the alimentary tract (Latimer *et al.*, 1990; Ramis *et al.*, 1994). The bursa of Fabricius also shows marked atrophy and necrotic tissue, causing immunosuppression in affected birds. Thus, the majority of birds do not tend to die from infection

with BFDV itself, but rather from secondary microbial infections (Latimer *et al.*, 1990; Latimer *et al.*, 1991a).

BFDV is highly contagious, and the viral antigen present in feathers and in many organs associated with the alimentary tract indicates that the virus can be spread horizontally through shedding of virus particles in feather dust, crop secretions, or faeces (Ritchie *et al.*, 1991a). Vertical transmission, from infected hens to embryonated eggs, has also been reported (Rahaus *et al.*, 2008). Within a captive environment, horizontal spread through environmental contamination is probably the most prevalent route of transmission.

While circoviruses affecting non-psittacine species have not been as extensively studied as BFDV, not all of them appear to cause feather dystrophy or loss, although they do appear to be lymphotropic, and therefore are also assumed to be immunosuppressive (Woods and Latimer, 2000).

1.4 Future for the family Circoviridae

1.4.1 New discoveries

With the advent of new sequencing technologies coupled with cheaper sequencing costs, a large diversity of circular ssDNA viruses has been discovered, indicating that there is much more diversity that initially thought. Viral metagenomics involves taking a sample from an environment, concentrating and purifying viral particles within the sample, extracting and amplifying the viral DNA, then shotgun sequencing the viral genomes or using next generation sequencing methods. Sequencing of circular ssDNA viruses has been greatly aided by the use of the bacteriophage phi29 (ϕ29) DNA polymerase in the non-specific amplification of circular DNA. This polymerase preferentially amplifies circular ssDNA, through a rolling circle amplification (RCA) method. Thus, viral metagenomic analyses have revealed the presence of viruses similar to circoviruses from a variety of different environments (Blinkova *et al.*, 2010; Ge *et al.*, 2011; Kim *et al.*, 2008; Li *et al.*, 2010a; Li *et al.*, 2010b; Rosario *et al.*, 2009a; Rosario *et al.*, 2009b; Victoria *et al.*, 2009). However, the majority of sequences isolated through metagenomic approaches are either completely unknown, or share low sequence homology with known viral proteins, indicating that there are perhaps undescribed species that could eventually be assigned to the *Circoviridae* family (Rosario and Breitbart, 2011). One such potential addition is the proposed genus *Cyclovirus* (Biagini, 2011b; Li *et al.*, 2010a). The first cyclovirus was identified from a metagenomic analysis of faecal samples from children, when a fragment of viral nucleic acid encoded a protein that showed close similarity to the

Rep protein of circoviruses (Victoria *et al.*, 2009). Sequencing of the full viral genome, however, indicated that it was different enough to be classified as a member of a new genus within the *Circoviridae* family, rather than simply a new species within the *Circovirus* genus, and the name *Cyclovirus* was proposed, from the Greek 'cyclo' meaning circular (Li *et al.*, 2010a). Using specific primers designed to anneal in the Rep genes of both known circoviruses and the newly identified cyclovirus, additional cyclovirus genomes were subsequently characterised from faecal samples from children and chimpanzees, as well as from a variety of samples from meat destined for human consumption (Li *et al.*, 2010a; Li *et al.*, 2011).

Inclusion of the genus *Cyclovirus* within the family *Circoviridae* is appropriate, as the circular ssDNA genome of cycloviruses has the same ambisense organisation as circoviruses, and the Rep proteins share similarities. However the genome is generally smaller than the circovirus genome, at 1.7-1.9kb, and correspondingly smaller ORFs encode the putative Rep and CP. The 5' intergenic region is generally larger than that of circoviruses, and the 3' intergenic region is either absent, or consists of only a few bases (Delwart and Li, 2012; Li *et al.*, 2010a; Li *et al.*, 2011). While the stem-loop structure in the 5' intergenic region seen in circoviruses is conserved in cycloviruses, the nonanucleotide sequence for initiation of rolling circle replication at the apex of the loop differs slightly from the circovirus sequence (cycloviruses, TAATACTAT; circoviruses, TAGTATTAC), and appears to be more highly conserved among known cyclovirus isolates, whereas the sequence is slightly more variable between circovirus species (Li *et al.*, 2010a; Li *et al.*, 2011). However the complementary strand of cycloviruses contains the recognised circoviral nonanucleotide motif TAGTATTAC, indicating that the cycloviruses may either use a different sequence as their origin of replication, or differ from circoviruses in having the Rep protein on a different strand to the nonanucleotide motif (Rosario *et al.*, 2012b). Since the original discovery, cycloviruses have also been discovered using metagenomic approaches in bat faecal matter and muscle tissue (Ge *et al.*, 2011; Li *et al.*, 2011; Li *et al.*, 2010b), and the abdomens of dragonflies (Rosario *et al.*, 2012a; Rosario *et al.*, 2011). When viruses are isolated through metagenomic approaches, it is not always possible to determine the natural host of the virus. The cycloviruses isolated from faecal matter may have been consumed in plant matter, animal tissue or insects, or they could represent a virus capable of enteric replication. Dragonflies may be natural hosts of cycloviruses, or they may have consumed insects that were infected with cycloviruses, or that had themselves consumed plant matter infected with cycloviruses. However, the fact that Rosario *et al.* (2011) documented these viruses in three species of dragonflies from three different islands of the Kingdom of Tonga and found evidence of recombination amongst these viral isolates, suggests that these may indeed infect dragonflies. That cycloviruses were isolated from

meat samples and from bat muscle tissue suggests that they are capable of replication within mammalian hosts. Further work will be necessary to determine conclusively if this is the case.

1.4.2 Taxonomic implications

As just mentioned, there is a proposal to include a new genus, *Cyclovirus*, within the Circoviridae family, although this has yet to be approved by the ICTV. The argument for inclusion is based on the shared similarities in genome organisation and structure, and homologous Rep proteins. However the genus *Gyrovirus* within the *Circoviridae* family containing CAV and potentially new species shows markedly different characteristics. As noted, while CAV shares some similarities with the circoviruses, there are also some major differences, such as a negative sense genome, a single polycistronic mRNA, and perhaps most importantly, lack of an obvious Rep protein. Recent discoveries of potentially novel gyroviruses that share similarities with CAV suggest that there are more gyrovirus species yet to be discovered. Thus there has been an alternative suggestion, that of reassigning gyroviruses to the family *Anelloviridae*, where they would be classified as a subfamily, *Gyrovirinae* (Biagini, 2011a). The *Anelloviridae* family currently contains nine genera, members of which infect human and nonhuman vertebrates, and share the circular negative sense ssDNA genome organisation observed in gyroviruses. These nine genera would then form a subfamily, named *Anellovirinae*. Given the similarities between CAV and anelloviruses, this would be appropriate. This would leave just the circoviruses and cycloviruses in the family *Circoviridae*. The proposal is to divide *Circoviridae* into two subfamilies; *Circovirinae*, which would encompass all current and any future members of the genus *Circovirus*, and *Cyclovirinae*, which would encompass members of the proposed genus *Cyclovirus*. Overall, when taking into consideration similarities and differences in genome organisation and structure, these suggested taxonomic alterations would appear to be appropriate (Biagini, 2011b).

1.5 Genetic Diversity of BFDV Isolates

Early on it was noted that different species had a tendency to manifest PBFD symptoms differently (Doneley, 2003; Kock *et al.*, 1993; Latimer *et al.*, 1991a; Schoemaker *et al.*, 2000). BFDV was assumed to have limited sequence diversity between virus isolates, as comparisons of ultrastructural characteristics, protein composition and antigenic reactivity indicated that BFDV isolates from four different psittacine genera were similar (Ritchie *et al.*, 1990), and the first two fully sequenced BFDV genomes from Australia and the USA had few major sequence differences (Bassami *et al.*, 1998;

Niagro *et al.*, 1998). However, following the isolation of PCV2 and the discovery that, unlike PCV1, it was pathogenic, the possibility that there were distinct genotypes of BFDV that preferentially infected certain psittacine species needed to be explored, as techniques developed to detect BFDV in one species might fail to detect it in another if the two strains were sufficiently different. Subsequently, an analysis based on the predicted amino acid sequences of the *rep* and *cp* genes of ten BFDV isolates from different species in Australia showed four main clusters of strains in the phylogenetic tree, indicating a greater genetic diversity than previously thought, although the authors could not attribute any regional or species specificity to the clusters (Bassami *et al.*, 2001). A phylogenetic tree based on the *rep* gene sequence of 17 new isolates from New Zealand and the ten isolates from the study by Bassami *et al.* (2001) displayed three distinct clusters infecting certain psittacine species, namely cockatoos, lorikeets and a budgerigar, with the budgerigar sequence being distinctly different from all other sequences (Ritchie *et al.*, 2003). A similar study using a portion of the *cp* gene from 31 species from different countries showed sequences from African Grey Parrots (*Psittacus erithacus*) with typical feather dystrophy, and from Rainbow Lorikeets (*Trichoglossus haematodus forsteni*) which recovered from an acute outbreak within an aviary, clustered in separate branches, indicating the possible existence of BFDV genotypes causing specific disease presentation (Raue *et al.*, 2004). Heath *et al.* (2004) found that African BFDV isolates were genetically different from those from other parts of the world, based on full genome sequences, and identified eight clusters in phylogenetic trees based on the full genome or *cp* gene sequences of African, Australasian, and American isolates, with African isolates forming at least three distinct genotypes. Other studies have suggested that if specificity exists, it is not absolute (de Kloet and de Kloet, 2004); that while cockatiels are rarely infected, BFDV isolates from cockatiels are serologically and genetically different from isolates from other species (Shearer *et al.*, 2008); and that there is a unique strain that infects budgerigars (Ogawa *et al.*, 2010; Varsani *et al.*, 2010).

Most of these studies involved comparisons between *rep* or *cp* gene sequences from BFDV isolates, yet these evolve at different rates, and, as is common among ssDNA viruses, circoviruses are subject to high levels of recombination, which provides the virus with a much greater means of exploring the available sequence space than mutation alone (Heath *et al.*, 2004; Lefeuvre *et al.*, 2009; Raue *et al.*, 2004). Therefore any phylogenetic analysis should be based on the full genome sequences of isolates, and include a recombination analysis to determine how virus strains are interacting.

One such study from 2011 analysed the full genome sequences of all isolates available in GenBank at the time, as well as 22 new isolates, for a total of 87 full BFDV genomes (Varsani *et al.*, 2011). After detailed analyses of pairwise identities of BFDV and other circoviruses it was suggested that BFDV

isolates that share 89-98% full genome sequence identity be considered different strains, and those sharing 98-100% sequence identity be considered subtypes of strains. The 87 isolates were therefore assigned to 14 different strains, designated A-N, with strains containing varying numbers of subtypes, denoted by a number in subscript (eg BFDV-A_1, BFDV-B_2). Interestingly, as was previously noted (Ogawa *et al.*, 2010; Ritchie *et al.*, 2003; Varsani *et al.*, 2010), full viral genome sequences from budgerigars were sufficiently different from other BFDV sequences to be considered a different species of circovirus, designated budgerigar circovirus (BCV), with its own strains and subtypes. From this study, it was clear that while some strains were isolated from only a single species or region, the majority of strains could be found in multiple species or areas. Most strains also showed evidence of recombination (Varsani *et al.*, 2011).

Thus the issue of whether or not any sort of species or regional specificity exists is not clear cut. It would appear that there are genetically diverse strains of BFDV, but they may not be limited to just one geographical area or species. One possible explanation for this is that while species remained in their natural ranges, there may have been time to develop regionally or species specific strains of BFDV through genetic drift, but once different species became mixed, either through introduction and subsequent establishment of wild populations of exotic species into new regions, or within captive facilities, the opportunity existed for different virus strains to recombine, blurring any lines of specificity that may have existed. The international trade in parrots, both through legal trade and illegal trafficking, has certainly contributed to this.

1.6 BFDV studies from around the world

1.6.1 BFDV in Australia

Although BFDV now has a global distribution, it is thought to have originated in Australia, and then spread to other countries through international trade in parrots. The natural diversity of psittacine species is greater in Australasia than in other regions that are home to parrots, with Australia having been referred to as the "land of parrots"(Forshaw, 2010). Perhaps the first description of what would later become known as psittacine beak and feather disease was in a letter to an Australian journal in 1907, in which the author notes a personal observation from 1887 of wild Red-rumped Parrots (*Psephotus haematonotus*) in the Adelaide hills being unable to fly, due to loss of all their feathers (Ashby, 1907). Another probable early case of PBFD infection was that of a captive Sulphur-crested Cockatoo in Sydney, named Cocky Bennett, a bird so well-known locally that his death in

1916 at the estimated age of 120 years old warranted an obituary in the local newspaper (Centre for Fortean Zoology Australia, 2011). Descriptions of Cocky from the early 1900's report that he was mostly featherless, with a severely elongated upper beak (Nicholls, 1914), classic symptoms of PBFD, and photographs from this time support the descriptions (Fig. 1.3).

Fig. 1.3. Cocky Bennet, an Australian Sulphur-crested Cockatoo thought to have BFDV, on his cage in 1914 at the approximate age of 118. The lack of feathers and elongated beak, typical symptoms of BFDV infection, are clearly visible. *Photo courtesy of Sutherland Shire Libraries.*

The name psittacine beak and feather disease was coined to describe a syndrome observed in the late 1970's, predominantly in captive psittacines in Australia, involving feather loss and abnormal growth of feathers and beak (Pass and Perry, 1984). The first official clinical investigation into the etiology and pathogenesis of the disease came in 1984, based on captive endemic Sulphur-crested Cockatoos (*Cacatua galerita*), Galahs (*Eolophus roseicapilla*) and Budgerigars (*Melopsittacus undulatus*), and captive exotic lovebirds (*Agapornis sp.*), all of which displayed symptoms (Pass and Perry, 1984). Viral-like inclusions were consistently noted in affected tissue from these birds, indicating that the etiological agent could be viral in origin. The same inclusions were noted in wild Sulphur-crested Cockatoos suffering from feather loss and beak abnormalities, and the suggestion

arose that a parvovirus was the cause (McOrist *et al.*, 1984), but an inability to grow the virus in tissue culture systems thwarted attempts to isolate and characterise it (Pass *et al.*, 1985).

Using an inoculum made by extracting viral particles from homogenates of affected feathers from naturally infected Sulphur-crested Cockatoos, Galahs, Little Corellas (*Cacatua sanguinea*), and a subspecies of the Port Lincoln Parrot (*Barnardius zonarius*) known as the Twenty-eight Parrot (*B. z. semitorquatus*), Wylie and Pass (1987) showed that disease symptoms identical to those observed in naturally infected birds could be experimentally reproduced in young Budgerigars and Galahs. Some of the control birds that were not inoculated but remained in contact with their inoculated nest mates also developed symptoms, further indicating the infectious nature of the disease (Wylie and Pass, 1987).

In an attempt to determine the prevalence of infection in the wild, a haemagglutination inhibition test was performed on individuals sampled from wild populations of native birds from three regions of New South Wales (Camden, Yeoval, and Mootwingee National Park). A high percentage of individuals from flocks of Sulphur-crested Cockatoos, Galahs, Little Corellas, Long-billed Corellas (*Cacatua tenuirostris*), and Mallee Ringneck Parrots (*Barnardius barnardi*) had antibodies to BFDV present in their blood serum, indicating that they had been exposed to the virus. Few individuals within the sampled flocks, however, displayed PBFD symptoms, thus the prevalence of infection in some species may be greater than that of disease (Raidal *et al.*, 1993c).

Sequencing and analysis of the first full BFDV genome in 1998 in Australia confirmed previous findings that the BFDV genome was circular, ssDNA of approximately 2kb (Bassami *et al.*, 1998). Analysis of the genome also revealed the similarities between BFDV, PCV and the circular ssDNA plant infecting viruses from the *Geminiviridae* and *Nanoviridae* families in the Rep. This full genome sequence was subsequently used to design PCR primers to amplify a portion of the *rep* gene, determined to be the least variable region of the genome, from all BFDV isolates (Ypelaar *et al.*, 1999). While useful as a means of rapidly diagnosing BFDV infections, the widespread use of these primers appears to have resulted in a lack of full BFDV genomes being sequenced. Despite an understanding of the need to sequence full genomes in order to establish phylogenetic relationships (Bassami *et al.*, 1998), only a further 11 full BFDV genome sequences have been published from Australia to date, ten of them from native species. Eight BFDV isolates from five captive and three wild birds (wild: two Sulphur-crested Cockatoos, one Galah; captive: one exotic Lovebird (*Agapornis roseicollis*), one Rainbow Lorikeet (*Trichoglossus haematodus*), one Major Mitchell's Cockatoo (*Cacatua leadbeateri*), one Bluebonnet (*Psephotus haematogaster*) and one Eastern Long-billed Corella) were sequenced in an effort to explore genetic diversity among isolates from different

species (Bassami *et al.*, 2001). Two full genome sequences from Cockatiels (*Nymphicus hollandicus*) confirmed the susceptibility of this species to infection, despite a lack of previous reports of infection (Shearer *et al.*, 2008). The final isolate, from a second Rainbow Lorikeet, was sequenced as part of a study into BFDV infection in New Caledonia, described in detail in Chapter three.

Given that a sub-set of the BFDV scientific community considers it to be endemic to Australia, the current paucity of data in the form of full length genome sequences is surprising; and most full genome datasets indicate otherwise. Knowledge of the strains currently present throughout the country would enhance our understanding of how the virus has evolved over time, potentially since its emergence. It would be beneficial to have full BFDV genome sequences from all endemic Australian psittacine species, both those in captivity and from the wild, as well as from any non-native psittacine species. Sampling on such a large scale would obviously be a massive undertaking, but the enhancement of current knowledge and the insights gained regarding the evolutionary history of BFDV would be invaluable.

1.6.2 BFDV in New Zealand

New Zealand has eight endemic psittacine species; three parrots, the Kea (*Nestor notabilis*), Kaka (*Nestor meridionalis*) and Kakapo (*Strigops habroptilus*), and five parakeets, the Red-fronted Parakeet (*Cyanoramphus novaezelandiae*), Yellow-crowned Parakeet (*C. auriceps*), Chatham Island Parakeet (*C. forbesi*), Orange-fronted Parakeet (*C. malherbi*), and Antipodes Island Parakeet (*C. unicolor*). It is also home to both captive and wild populations of exotic parrot species.

While major studies into the prevalence of BFDV in New Zealand did not occur until early in the twenty-first century, there are indications that the virus was present well before then. In 1985 two wild Eastern Rosellas were captured in Warkworth, in the upper North Island, suffering from feather loss. Microscopic examination indicated a viral infection within the epidermal cells (Julian and McKenzie, 1985). Red-fronted and Yellow-crowned Parakeets kept in captivity on bird reserves during the 1980's were reported to suffer from a disease causing anaemia and occasional death, with loss of primary tail feathers, and similar symptoms were also described in wild populations of Yellow-crowned Parakeets in Te Anau (Vickers, 1991).

Investigation into BFDV in exotic species demonstrated infection among wild populations of Sulphur-crested Cockatoos (*Cacatua galerita*) and Eastern Rosellas (*Platycercus eximius*), some of which tested positive through polymerase chain reaction (PCR) despite showing no external symptoms of infection (Ha *et al.*, 2007). Infection has also been reported in captive Sulphur-crested Cockatoos, along with Rainbow Lorikeets (*Trichoglossus haematodus*), Yellow-bibbed Lorikeets (*Lorius*

chlorocercus), a Red-collared Lorikeet (*Trichoglossus haematodus rubritorquis*), a Goldie's Lorikeet (*Psitteuteles goldiei*), Blue-streak Lorikeets (*Eos reticulate*), Longbilled Corellas (*Cacatua tenirostitus*), and Budgerigars (*Melopsittacus undulatus*) (Ritchie *et al.*, 2003). As all eight of New Zealand's native parrots are considered threatened, and as such are the focus of conservation management plans, there exists a need to assess susceptibility of the native parrots to infection with BFDV, and to determine transmission of the virus among native and exotic psittaciformes.

An initial study involving 312 wild and captive native birds from different species found that four captive birds were BFDV positive, two Red-fronted Parakeets and two Antipodes Island Parakeets, but no wild birds tested positive, leading to the conclusion that while native birds, and in particular native parakeets, appeared to be susceptible to infection, the true prevalence of infection in the wild was very low, with estimates ranging from <4% to <7% in different species (Ha *et al.*, 2009). However another study focusing on a wild population of Red-fronted Parakeets on Little Barrier Island, a predator-free island sanctuary, found the prevalence of infection to be a much higher 28% (Ortiz-Catedral *et al.*, 2009). Following on from this study, the full BFDV genomes isolated from five Red-fronted Parakeets were sequenced and compared to all full BFDV genome sequences available in GenBank in order to determine the evolutionary relationships between New Zealand virus isolates and isolates from the rest of the world. From this it was found that the virus infecting New Zealand's Red-fronted Parakeets is a unique strain of BFDV, not having been sampled from any other species anywhere in the world (Ortiz-Catedral *et al.*, 2010). This strain was later designated BFDV-A_1 (Varsani *et al.*, 2011).

More recently, the most comprehensive study into BFDV ever to have been undertaken in New Zealand was completed (Massaro *et al.*, 2012). The purpose of this study was not only to systematically screen both captive and wild populations of native birds from multiple sites throughout the country for BFDV, but also to fully sequence the virus isolates from positive samples and compare these sequences to full viral genome sequences isolated from species introduced to New Zealand, as well as from species worldwide. This comparison would help to increase the understanding of virus transmission in New Zealand, to determine whether the native birds were being infected by the introduced species, or whether the introduced species were being infected with New Zealand's own unique strain. From a total of 786 birds sampled (753 native, 33 Eastern Rosellas), 16 Red-fronted Parakeets, eight Yellow-crowned Parakeets, and seven Eastern Rosellas were found to be positive for BFDV. Massaro *et al.* (2012) not only confirmed BFDV infection in the Red-fronted Parakeet, but discovered infection within a population of Yellow-crowned Parakeets, the first time this species had ever been found to have BFDV, and the first discovery of BFDV in the

South Island (described in detail as part of this thesis; chapter two), although indications are it may have existed for some time within this population (Vickers, 1991). Phylogenetic analysis of the full genome viral isolates once again assigned the Red-fronted Parakeet isolates to the BFDV-A_1 strain and subtype, with the Eastern Rosella isolates being the same strain, but different subtypes (BFDV-A_2-A_6; see Varsani *et al.* (2011) for a description of species/strain/subtype demarcation).

1.6.3 BFDV in New Caledonia (Nouvelle-Calédonie)

Located in the south-west Pacific Ocean, the French territory of New Caledonia is comprised of a group of islands approximately 1200km east of Australia and 1700km north of New Zealand. It is home to three endemic psittacine species, the Horned Parakeet (*Eunymphicus cornutus*), the Ouvéa Parakeet (*E. ouveaensis*), and the New Caledonian Parakeet (*Cyanoramphus saisetti*), all of which are considered threatened. While there have been no confirmed sightings since 1913 of a fourth endemic species, the New Caledonian Lorikeet (*Charmosyna diadema*), it is still not officially classified as extinct, but is considered critically endangered, with unconfirmed sightings reported in the 1950s and in 1976. The Rainbow Lorikeet (*Trichoglossus haematodus*) has up to 20 recognised subspecies, based on differences in colouring (Forshaw, 2010). One subspecies, the Deplanche's Lorikeet (*T. haematodus deplanchii*), is found only in New Caledonia, although it is not considered threatened. New Caledonia also has various exotic parrot species in zoos or captive breeding facilities, providing a potential route for the virus to enter the country. Prior to the study reported later in this thesis, there have been no reports of BFDV in New Caledonia.

1.6.4 BFDV in the Americas

Following the extinction of the Carolina Parakeet (*Conuropsis carolinensis*), presumed to have occurred around the 1930's, North America has no endemic parrots (Forshaw, 2010). Yet, as is the case for most countries, many exotic psittacine species have been imported over the years for aviculture and pet trade, particularly from South America, and there are reports of exotic parrots establishing wild populations following escape or release from captivity (Bull, 1973; Butler, 2005). Following the description of PBFD in Old World psittacine species in 1984 (Pass and Perry, 1984), the first cases of PBFD in New World psittacine species were described in America, from a Blue-fronted Amazon (*Amazona aestiva*) (Huff *et al.*, 1988) a Red-lored Amazon (*Amazona autumnalis*) (Ritchie *et al.*, 1990), and a Scarlet Macaw (*Ara macao*) (Greenacre *et al.*, 1992), confirming the susceptibility of New World species to infection.

BFDV was finally characterised by isolating and purifying viral particles from diseased tissue, after repeated attempts to grow the virus in *in vitro* cell culture had failed. Following this, intensive study

into various aspects of BFDV and PBFD occurred in America. Latimer *et al.* (1990) confirmed that inclusion bodies repeatedly observed in the tissues of PBFD affected birds contained BFDV, as did feather inclusions (Latimer *et al.*, 1991b). The dynamics of viral shedding from infected birds was studied, indicating potential routes of infection (Ritchie *et al.*, 1991a). BFDV was discovered to agglutinate some red blood cells (RBCs), enabling the development of haemagglutination and haemagglutination-inhibition tests for rapid diagnosis of BFDV infection (Ritchie *et al.*, 1991b), and reports of secondary parasitic and microbial infections in birds infected with BFDV provided additional evidence of the immunosuppressive nature of the virus (Latimer *et al.*, 1993; Latimer *et al.*, 1996; Latimer *et al.*, 1990; Latimer *et al.*, 1992).

In the same year as the sequence of the first full BFDV genome in Australia was determined (Bassami *et al.*, 1998), another isolate was sequenced in the United States, derived from pooled blood from infected birds (Niagro *et al.*, 1998). This sequence consequently cannot be assigned to any one species. Despite the considerable contribution studies from America have made to the general knowledge base regarding BFDV, there has only ever been one other full BFDV genome to have been sequenced from America, from virus isolated from a Ring-necked Parakeet (*Psittacula krameri*) in Texas (de Kloet and de Kloet, 2004).

1.6.5 BFDV in Africa

In 2002, Bragg (2002) commented that the feather disorder noted among Budgerigar breeders in South Africa and attributed to Avian Polyomavirus (APV), may instead be the result of BFDV infection. This had been previously documented in captive populations of lovebirds in Zimbabwe, where an outbreak within an aviary resulted in 100% mortality in two native species of lovebird, the Black-cheeked Lovebird (*Agapornis nigrigensis*) and the Lilian's or Nyasa Lovebird (*A. lilianae*), while leaving Peach-faced (*A. roseicollis*) and Fischer (*A. fischeri*) Lovebirds relatively unaffected (Kock *et al.*, 1993). BFDV was later found in captive bred Ring-necked Parakeets (*Psittacula krameri*) and Budgerigars (*Melopsittacus undulatus*) in South Africa, which were exhibiting feather disorders (Albertyn *et al.*, 2004). Restriction digest profiles of virus isolates from budgerigars from the Craddock region, obtained by digesting the PCR amplified region of the BFDV ORF 1 with the restriction enzyme *Hae*III, were different from restriction digest profiles from Pretorian budgerigars, indicating the possibility of genetically different BFDV isolates in the two areas, and that one bird was potentially infected with both isolates (Albertyn *et al.*, 2004).

Heath *et al.* (2004) examined the genetic relationship between ten full length genome sequences from South African BFDV isolates from various species and ten full length BFDV genome isolates

from Australia and the US. Three of the South African isolates were closely related to the US BFDV sequence obtained from pooled blood, while the rest of the isolates formed three distinct clusters, indicating that South African isolates are genetically divergent from Australian isolates. The degree of genetic diversity between South African isolates also suggested that the virus had probably been present in Africa for some time, rather than being a recent introduction (Heath *et al.*, 2004).

Kondiah *et al.* (2006) found different genotypes of BFDV based on the *Rep* gene sequence, including a specific budgerigar lineage, but did not perform full genome analyses. Consequently, Varsani *et al.* (2010) characterised the full BFDV genomes from budgerigars from the same breeding facility, and upon comparison with all full length BFDV sequences in the GenBank database, as well as the Rep and CP sequences, suggested that the budgerigar isolate could represent a BFDV strain unique to budgerigars worldwide (Varsani *et al.*, 2010).

The presence of BFDV in Africa has increased the threat to survival faced by endangered parrots, most notably the South African Cape Parrot (*Poicephalus robustus*) and the Echo Parakeet (*Psittacula echo*) on the island of Mauritius. BFDV has been reported in both these species, and has been implicated both as a contributing factor to the declining numbers of Cape Parrots in the wild (Wirminghaus *et al.*, 1999) and an impediment to the intensively managed recovery programme for the Echo Parakeet (Kundu *et al.*, 2012).

1.6.6 BFDV in Asia

BFDV has been detected multiple times in various regions of Asia over the last decade. The first cases of BFDV infections in Thailand occurred in three captive Sulphur-crested Cockatoos imported from Indonesia, which presented with feather loss. PCR testing confirmed the presence of BFDV DNA, but no sequencing or phylogenetic analysis was done (Kiatipattanasakul-Banlunara *et al.*, 2002). Over a two year period, from 2005-2006, feather and blood samples were taken from various psittacine species in Thailand to be tested for BFDV. Seventeen samples from seven different genera tested positive, and the full length BFDV genomes from these samples were isolated, sequenced and analysed (Sariya *et al.*, 2011). The full length genomes were found to be similar, ranging from 91-100% identity, and phylogenetic analysis of the predicted amino acid sequence of the CP from the Thailand isolates and other published CP amino acid sequences formed ten clusters, with the Thailand isolates occurring in three clusters. Cluster I was made up entirely of Thailand isolates (12 sequences), cluster II was made up of four Thailand sequences and one from Australia, and the final Thailand isolate, from a lovebird (*Agapornis sp*), was grouped in cluster V along with isolates from Australia, USA and the UK (Sariya *et al.*, 2011). Many of the sequences, particularly those from group

I, showed little sequence diversity, indicating a recent infection, and the closeness to Australian sequences suggests that the virus may have arrived in Thailand from Australia, and quickly spread through a captive facility, infecting susceptible birds.

A three year study to determine the prevalence of BFDV infection in Taiwan found a 41.2% BFDV positive rate, and dual infections with Avian Polyomavirus (APV) were also noted, at a prevalence of 10.3% (Hsu *et al.*, 2006). However no full genome sequences from any of the positive samples were isolated and phylogenetic analyses were based on the Rep and CP nucleotide sequences.

Following the isolation and characterisation of BFDV from two cockatiels in Australia (Shearer *et al.*, 2008), a third full genome sequence was isolated from an infected cockatiel in Japan (Katoh *et al.*, 2010). Phylogenetic analyses based on the nucleotide sequences of Rep and CP placed the Japanese cockatiel isolate with other Australian *Cacatuidae* isolates, including the two cockatiel sequences, but no full genome comparisons were made. Six BFDV isolates from budgerigars in Japan formed two clusters, distinct from each other and separated from all other isolates, based on the full genome nucleotide sequences (Ogawa *et al.*, 2010), and a single full genome sequence isolated from China clustered with one of the Japanese groups (Zhuang *et al.*, 2012), which potentially indicates a separate species of circovirus specific to budgerigars (Varsani *et al.*, 2011) or possibly divergent BFDV strains.

While not all Asian studies necessarily used the full BFDV genome sequences in their phylogenetic analyses, at least they were sequenced and added to the database GenBank, to enable their use in other studies.

1.6.7 BFDV in Europe

While the natural range of most wild psittacine birds is in tropical or subtropical regions, predominantly in the Southern hemisphere, they are also known to naturally inhabit more temperate regions, such as areas of South America and the islands of New Zealand (Forshaw, 2010). Europe has no native psittacine species, yet the popularity of the species as pets meant Europe was one of the most prolific importers of wild-caught exotic parrots for the pet trade or personal collection, until the practice was banned in 2007 (Commission Regulation (EC) No. 318/2007). While the practice was legal, there were many instances of accidental or intentional release of birds to the wild, which has led to the establishment of wild populations of exotic psittacines, at least in the more temperate regions of Europe (Chiron *et al.*, 2009; Muñoz and Real, 2006; Strubbe and Matthysen, 2009). As such, psittacine species now account for approximately 18% of Europe's established wild populations of exotic birds (Strubbe and Matthysen, 2009). Therefore there is a high

probability that virus carrying birds have been imported into the country at some stage, probably multiple times, and a possibility that BFDV exists in the established wild populations of psittacines, as well as within breeding facilities. It is also highly likely that illegal trafficking of exotic parrots into Europe still exists to some degree, and as this is unregulated, poses a higher risk of introducing diseased birds into the country. Despite the ban on importing exotic parrots, established European breeding facilities are still able to supply birds for the pet trade within Europe, as well as export their birds to countries outside of Europe. In the absence of rigorous testing programmes to ensure these birds are free from disease, there is a risk of selling or exporting birds infected with BFDV.

Rahaus and Wolff (2003) conducted one of the first studies into the prevalence of BFDV in captive psittacines in Europe. They tested 146 birds with no previous history or current symptoms of PBFD, from 32 breeders throughout Germany. Approximately 40% of the birds tested positive for BFDV through PCR, indicating a high rate of BFDV infection in captive facilities in Germany. The authors surmised that due to trading of captive bred psittacine birds throughout Europe, there would be a similarly high rate of infection in other European countries. The fact that none of the birds that tested positive showed any symptoms of PBFD clearly indicates the need for testing programmes, as seemingly healthy birds which are sold or exported could actually be infected, thus contributing to the spread of the virus.

A larger study, consisting of 1516 birds from 18 breeding centres, four trade centres, and 36 private owners, tested over a period of four years, was conducted in Italy (Bert *et al.*, 2005). This study found a lower rate of infection, at 122 positive out of 1516, or approximately 8%, although the authors cautioned that the results were not comparable to the German study due to differences in sample size and type, namely the Italian study used blood samples, while the German study used feather samples. While it has been noted that feather samples may be the more reliable indicator of BFDV infection, with or without the presence of clinical symptoms (Hess *et al.*, 2004), this is not necessarily agreed with (Khalesi *et al.*, 2005). While the majority of birds testing positive were asymptomatic, 21 displayed characteristic symptoms. A similar large study in Poland involving 751 birds from private breeders also used feather samples, as did Rahaus and Wolff (2003), and found a similarly high rate of infection, at 25.3% (Piasecki and Wieliczko, 2010).

These three main studies into the prevalence of BFDV in Europe were based on PCR amplification of a 717bp fragment of ORF1, or the Rep gene, as described by Ypelaar *et al.* (1999), to determine presence or absence of infection. In contrast, when BFDV was detected in six deceased African Grey Parrots in Portugal, the full viral genomes were isolated and sequenced (Henriques *et al.*, 2010). Sequencing of a fragment of ORF1 reveals little about the evolutionary history of the genome as a

whole, and as circoviruses have been shown to be highly recombinant, with recombination hotspots located on the C-terminus of CP and in the intergenic region (Lefeuvre *et al.*, 2009; Varsani *et al.*, 2011), any analysis based on the ORF1 fragment would reveal very little about potential recombination events. Full genome analyses allow the detection of any recombination events, can provide information on which strains are involved in recombination, and can also be used to track the source of infections.

Thus it is probable that there have been multiple introductions of BFDV into Europe, and the unregulated trading of birds throughout European countries enables the spread of the virus, as well as recombination between different strains. The lack of symptoms in many birds that tested positive highlights the risk of transporting birds to new environments without first ascertaining whether or not they carry the virus.

1.7 BFDV infections: Diagnosis, control, and implications for conservation

1.7.1 Methods for detecting BFDV

The discovery that BFDV could agglutinate erythrocytes from some psittacine species allowed the development of a haemagglutination (HA) test to demonstrate infection with BFDV, and to quantify the amount of virus present in a sample (Raidal and Cross, 1994; Raidal *et al.*, 1993a; Ritchie *et al.*, 1991b). Haemagglutination inhibition (HI) assays can correspondingly be used to detect antibodies to BFDV, which can be useful in diagnosing clinically normal birds that have mounted an immune response following exposure to the virus (Raidal *et al.*, 1993a; Ritchie *et al.*, 1991b).

Once the BFDV genome was fully sequenced (Bassami *et al.*, 1998; Niagro *et al.*, 1998), primers designed to bind to and amplify a portion of the *rep* gene were developed (Ypelaar *et al.*, 1999), and later refined (Ritchie *et al.*, 2003), to enable diagnosis of BFDV through the polymerase chain reaction (PCR). Of the available tests, PCR has proven to be more specific and have greater sensitivity (Khalesi *et al.*, 2005). The development of a quantitative real-time PCR (qPCR) assay (Shearer *et al.*, 2009) allowed simultaneous detection of BFDV and quantification of the amount of virus present in a sample, which cannot be achieved through standard PCR.

More recently, the use of multiple random primers along with the bacteriophage ϕ29 DNA polymerase, which amplifies circular DNA through a rolling circle amplification (RCA) process, has enabled the rapid isolation of viral circular DNA genomes (Johne *et al.*, 2009; Shepherd *et al.*, 2008).

This technique has been successfully applied to BFDV positive samples (Ortiz-Catedral *et al.*, 2010; Varsani *et al.*, 2010; Varsani *et al.*, 2011) allowing the rapid isolation, cloning, and sequencing of full BFDV genomes. The sequencing of a portion of the BFDV genome is not sufficient to infer phylogenies, so any sample PCR positive for BFDV should subsequently be amplified by RCA and sequenced, not only to confirm the presence of BFDV, but so that the full genome can be analysed to establish appropriate phylogenetic relationships between isolates.

1.7.2 Methods of controlling BFDV transmission

Control of BFDV in the wild is virtually impossible, due to the stable nature of the virus. However prevention of transmission within the captive environment is achievable, for the most part through vigilant screening of existing birds, and appropriate quarantine measures and testing of any new birds before they are permitted to join existing populations. There is currently no cure for BFDV, nor any effective treatment measures, although the use of interferon and β-(1,3/1,6)-D-Glucan in treating BFDV have been reported (Stanford, 2004; Tomasek and Tukac, 2007). Infected birds can be kept in a warm, draught-free area to assist with thermoregulation once feathers are lost, and kept isolated so as to prevent spreading the virus to healthy birds, and to reduce the chances of contracting a secondary infection, which is more often the cause of death in infected birds (Latimer *et al.*, 1990; Latimer *et al.*, 1991a). The most logical solution would be to use a vaccine within the captive environment to prevent viral transmission, and which could be administered to any birds that are to be released from captivity to the wild, or translocated from one area to another, to prevent contracting the virus in the wild. Purified, inactivated virus from chronically diseased birds was experimentally used as a vaccine, and inoculated birds developed antibodies to BFDV, while chicks hatched from vaccinated hens were temporarily resistant to challenge with BFDV (Raidal *et al.*, 1993b; Ritchie *et al.*, 1992). However the inability to grow BFDV in cell culture means there is no way of determining the efficacy of inactivation methods *in vitro*, thus the danger exists of using an improperly inactivated virus and infecting birds, rather than protecting them. It also means that large quantities of the virus, needed for vaccine development, cannot be produced *in vitro*, and the ethics of keeping a supply of chronically infected birds purely to provide BFDV antigen for vaccine production are questionable at best. Consequently, at present, there is no vaccine available, although attempts to produce a safe effective vaccine are ongoing (Bonne *et al.*, 2009).

1.7.3 Implications of BFDV infections for conservation strategies

Psittacine species are considered to be one of the most threatened groups of birds worldwide, with 149, or nearly 42%, of the 356 species currently recognised categorised as near threatened to

critically endangered (BirdLife International, 2012). Thus, the global distribution of a virus as contagious and potentially fatal as BFDV poses a serious threat to the future of some psittacine species.

Due to their status as at risk of extinction, many psittacine species are the subject of intensive conservation management strategies. These strategies may involve breeding individuals in captive facilities such as zoos, or in private aviaries, with the aim of eventually releasing birds to the wild to boost the numbers in existing wild populations (Wilkinson, 2000). While such strategies can be advantageous in conservation of and public education regarding endangered species, and keep endangered species safe from predation, they do have inherent issues (Snyder *et al.*, 1996). The stress of confinement can potentially lower the immune system, rendering the birds more susceptible to infection. As well as this, having multiple exotic species in close proximity when they would be highly unlikely to encounter each other in the wild exposes BFDV naïve birds to infection, or enables evolution of the virus through recombination of different strains, potentially leading to the emergence of new strains with greater infectivity.

Another conservation strategy is that of translocating individuals from an existing population to a suitable new area, usually a predator-free island, where they can establish a new wild population with close monitoring by conservation staff (Jones, 2004). However although the island may be guaranteed pest-free, if it is or has been home to parrots in the past, or is within flying distance of parrots from the mainland or other islands, then it cannot be guaranteed BFDV free.

Therefore the establishment of comprehensive screening programmes is absolutely essential before transferring any birds from the wild to a captive environment, to avoid introducing BFDV into an aviary where it can spread among other birds. An outbreak in an aviary is notoriously difficult to treat, as BFDV is so environmentally stable, and resistant to usual methods of control. Symptoms are not always visible, and one infected bird could result in infection of an entire aviary. If feed dishes, perches, toys or other equipment are transferred without first being properly disinfected, it could cause a fresh outbreak in a new aviary. Carers for the birds could also inadvertently spread the virus through contaminated feather dust or faeces on their clothing or shoes as they move from aviary to aviary.

Any screening programme also needs to include any birds being translocated, in order to avoid introducing BFDV to a new area. Care also needs to be taken by staff involved in monitoring the progress of the new population, so that they do not introduce the virus through contaminated equipment. Human intervention, while necessary in managing the recovery of endangered species,

has been implicated as a contributing factor in the spread of BFDV among managed wild populations of the endangered Mauritius Parakeet (*Psittacula echo*) (Kundu *et al.*, 2012).

The international trade in exotic psittacines has resulted in the global distribution of BFDV, placing vulnerable psittacine species at risk of extinction. Legal trade of psittacine species needs to be highly regulated, with screening regimes and appropriate quarantine measures implemented, to prevent introducing BFDV into areas where birds may not have encountered the virus, or to prevent introduction of a different strain that could recombine with an existing strain. Greater measures also need to be taken to prevent illegal trade in wild caught psittacines, as this unregulated form of trade increases the risk of spreading the virus.

Even with the most stringent measures in place to prevent accidental introduction of BFDV to new environments, the worst could still occur. Thus, any conservation programme needs to have not only a comprehensive testing regime in place, but also a risk management plan for dealing with an infection should it occur, in order to minimise the spread of the virus and prevent a potentially fatal outbreak. At all costs, the most vulnerable, critically endangered psittacine species should be protected.

Chapter two: First discovery and Molecular Characterisation of BFDV in the South Island of New Zealand

Abstract

Beak and feather disease virus, the causative agent behind psittacine beak and feather disease, an often fatal disease affecting parrot species, has recently been discovered in a wild population of one of New Zealand's native parrots, the vulnerable Red-fronted Parakeet (*Cyanoramphus novaezelandiae*). This discovery instigated a large scale investigation into some of New Zealand's other native parrot species, to determine whether they too were infected. As part of this study, I was mainly responsible for testing Yellow-crowned Parakeet samples from the South Island. A total of 788 birds from seven endemic species and one exotic species, the Eastern Rosella (*Platycercus eximius*), were tested. BFDV was found in the North Island in wild populations of Red-fronted Parakeets, with a prevalence of 10.5%, and in Eastern Rosellas, with a prevalence of 21.9%. For the first time, BFDV was also found in the South Island, in a wild population of the Yellow-crowned Parakeet (*C. auriceps*), with a prevalence of 26.7%. This study provides evidence that more than one strain of BFDV is present in New Zealand, and highlights the need for continuous screening of both wild and captive birds, in order to limit the risk of transmission to New Zealand's other endangered parrots.

2.1 Introduction

The 2008 discovery of a unique strain of beak and feather disease virus (BFDV) in wild populations of Red-fronted Parakeets (*Cyanoramphus novaezelandiae*) on Little Barrier Island, off the coast of the North Island of New Zealand, raised the question of whether other native parrots were similarly infected (Ortiz-Catedral *et al.*, 2010; Ortiz-Catedral *et al.*, 2009). As all of New Zealand's native parrot species are considered to be at risk of extinction to a greater or lesser degree, it is important to determine the BFDV status of wild populations, as this can have an impact on conservation management decisions. Wild populations of introduced species such as the Eastern Rosella (*Platycercus eximius*) and the Sulphur-crested Cockatoo (*Cacatua galerita*) had previously been shown to be infected (Ha *et al.*, 2007), but a lack of full BFDV genome sequences from infected exotic birds meant it was not possible to determine whether exotic and endemic species were infected with the same strain of BFDV, or whether the virus has been transmitted from exotic species to endemic, or vice versa. In order to address these issues, a large coordinated investigation into the prevalence of BFDV in New Zealand began, involving sampling individuals from populations of seven native species, along with populations of the introduced Eastern Rosella. Samples were taken from birds in the wild and in captive environments. The native species sampled were Kakapo (*Strigops habroptila*), Malherbe's Parakeet (*Cyanoramphus malherbi*), Kaka (*Nestor meridionalis*), Chatham Parakeet (*Cyanoramphus forbesi*), Kea (*Nestor notabilis*), Yellow-crowned Parakeet (*Cyanoramphus auriceps*), and Red-fronted Parakeet, and sampling sites spanned the length of New Zealand, including offshore islands (Fig. 2.1). As part of this study, I was involved in testing South Island Yellow-crowned Parakeet samples for BFDV (Table 2.1). Full details of this study have been published in the Journal Archives of Virology (Massaro *et al.*, 2012).

2.2 Materials and methods

2.2.1 Sample collection, DNA extraction, and PCR screening for BFDV.

Blood and/or feather samples were obtained from a total of 788 birds from various regions in New Zealand (Fig. 2.1). South Island samples were collected by Jason van de Wetering, Maddie van de Wetering, and Moira Pryde of the Department of Conservation (DOC). New Zealand wide sample collection was coordinated by Kate McInnes, also of DOC. Whole blood, collected by venipuncture of the brachial vein, was stored in Queens Lysis Buffer (10 mM Tris, 10 mM NaCl, 10 mM disodium-EDTA, 1 % n-lauroylsarcosine, pH 8.0). Either 2mm of the tip of the quill (calamus) cut from the

feather using a sterile scalpel, or 10µl of blood in Queens Lysis Buffer was added to 10µl of 'lysis solution' from the Extract-n-Amp™ Blood PCR Kit (Sigma-Aldrich, USA) and incubated for 10 minutes at room temperature, after which 90µl of 'neutralisation solution' was added to yield crude total DNA. One µl of crude DNA was used in a PCR reaction with KAPA Blood PCR Kit Mix B (KAPA Biosystems, South Africa) according to the manufacturer's protocols, and the primers 5'-TTA ACA ACC CTA CAG ACG GCG A-3' and 5'-GGC GGA GCA TCT CGC AAT AAG-3', which amplify a 605-bp segment of the rep gene of BFDV (Ritchie *et al.*, 2003). The PCR protocol was as follows: an initial denaturation step of 94°C for 5 min, followed by 25 cycles of 94°C for 30 s, 56°C for 30 s and 72°C for 45 s, with a final extension step of 72°C for 1 min and cooling to 4°C for 10 min. The PCR products were resolved on a 1% agarose gel stained with SYBR® Safe DNA stain.

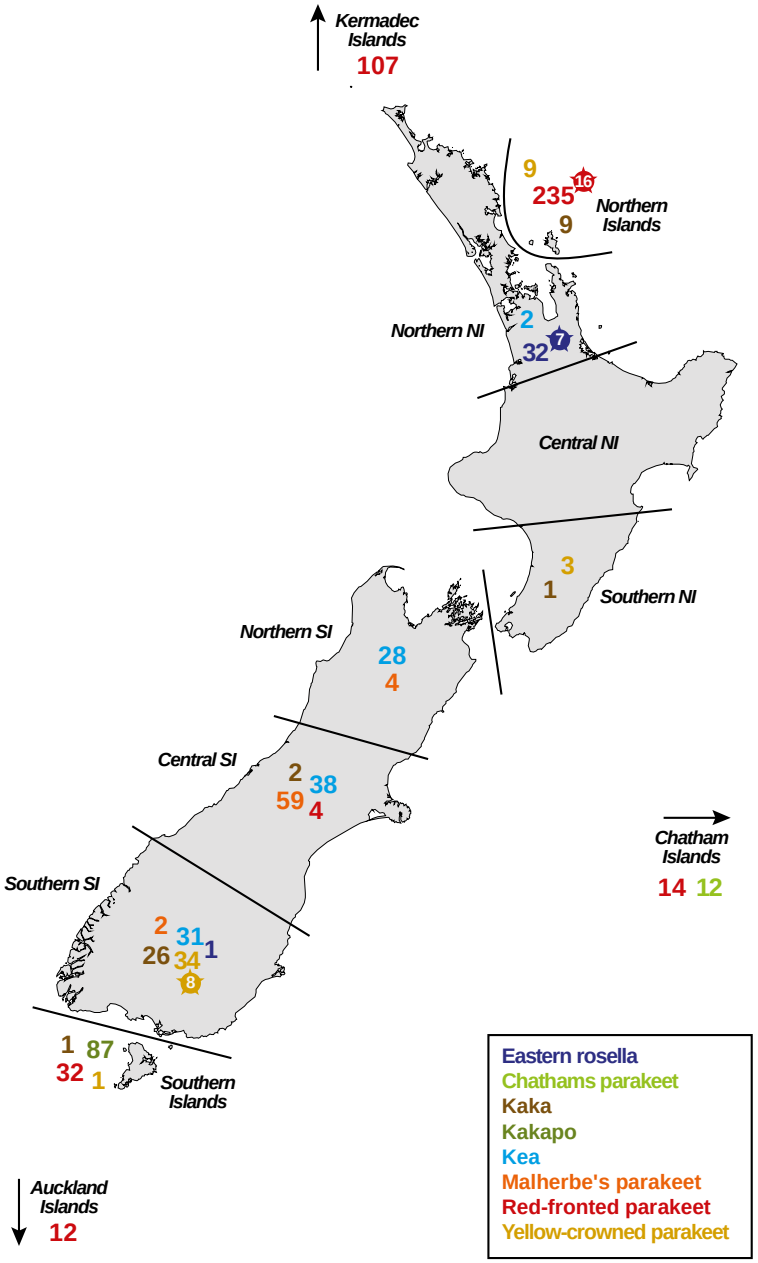


Fig. 2.1. Map of New Zealand, showing distribution of sampling sites and number of birds tested at each site. Numbers in coloured symbols (⬟) indicate the number of BFDV positive samples from that site.

Table 2.1. List of species tested for BFDV and sites sampled in New Zealand. Yellow-crowned Parakeet samples processed and tested by myself are indicated in bold red type. *Captive [a] 2008, [b] 2009; [c] 2010; [d] 2011; [e] 2012

Species	Common name	Population size tested	Wild	Captive	Location (sample numbers)	Sampling year (sample numbers)	BFDV positive
Nestor meridionalis	Kaka	39	37	2	Poteriteri (4) Isaac's Trust (2)* Eglinton Valley (19) Little Barrier Island (8) Great Barrier Island (1) Codfish Island (1) Karori Sanctuary (1) Waitutu forest (3)	2010 (4) 2010 (2) 2004 (7); 2010 (1); 2011 (11) 2008 (1); 2009 (4); 2010 (3) 2010 (1) 2010 (1) 2009 (1) 2004 (3)	0
Strigops habroptilus	Kakapo	87	87	0	Codfish Island (87)	2009 (1) 2010 (86)	0
Nestor notabilis	Kea	99	95	5	Arthur's Pass (2) Dana Bivvy (4) North Fiordland (16) Kahurangi (9) South Fiordland (5) Lewis Pass (5) Lake Rotoiti (11) Central Fiordland (6) Waimakariri (22) Westland (14) Auckland Zoo (2)* Naturelands (3)*	2010 (2) 2006 (4) 2005 (2); 2010 (14) 2009 (8); 2010 (1) 2006 (4), 2010 (1) 2009 (5) 2009 (11) 2006 (4), 2010 (2) 2009 (4); 2010 (18) 2008 (4); 2009 (10) 2010 (2) 2010 (3)	0
Cyanoramphus malherbi	Orange-fronted Parakeet	65	6	59	Isaac's Trust (59)* Chalky Island (2) Maud Island (4)	2008 (17); 2009 (17); 2010 (25) 2010 (2) 2010 (4)	0
Cyanoramphus novaezelandiae	Red-fronted Parakeet	404	400	4	Isaac's Trust (3)* Adams Island (12) Willowbank (1)* Codfish Island (32) Little barrier Island (153) Rangatira Island (14) Raoul Island (107) Tiritiri Matangi Island (82)	2010 (3) 1999 (12) 2009 (1) 2008 (12); 2010 (20) 2008 (71); 2009 (46); 2010 (36) 2009 (14) 2008 (78) 2010 (29) 2004 (1); 2005 (10); 2007 (29); 2008 (2); 2009 (23); 2010 (17)	14[a]; 1[b]; 1[c]
Cyanoramphus auriceps	Yellow-crowned Parakeets	47	43	4	Mana Island (3) Codfish Island (1) Little barrier Island (9) Invercargill (4)* **Eglinton (30)**	2005 (3) 2010 (1) 2010 (9) 2010 (4) **2011 (1); 2012 (29)**	**1[d], 7[e]**
Cyanoramphus forbesi	Chathams Parakeet	12	12	0	Mangere Island (12)	2009 (12)	0
Platycercus eximius	Eastern Rosella	33	31	2	Auckland region (32) Dunedin (1)	2009 (3); 2010(29) 2012 (1)	1[b], 6[c]

2.2.2 Isolation and cloning of full BFDV genomes using rolling circle amplification (RCA).

Samples testing positive for the rep gene of BFDV through the initial PCR screening method had the whole genome amplified through rolling circle amplification (RCA) using TempliPhi™ (GE Healthcare, USA) as described previously (Ortiz-Catedral *et al.*, 2010; Shepherd *et al.*, 2008; Varsani *et al.*, 2010; Varsani *et al.*, 2011). Briefly, 1µl of crude DNA extract was added to 4µl of TempliPhi™ sample buffer, heated for 2 min at 94°C, and cooled to room temperature. Five microlitres of reaction buffer and 0.2µl of TempliPhi™ enzyme mix (containing ϕ29 DNA polymerase) were added, and the nonspecific amplification reaction was allowed to continue at 30°C for 20 hours. The resulting concatemers were digested with *Bam*HI to produce full length BFDV genomes, and the ~2kb bands resolved on a 0.7% agarose gel were excised, cleaned using DNA Clean & Concentrator™-5 (Zymo Research, USA), ligated into a *Bam*HI-digested pUC19 plasmid vector, and sequenced by primer walking at Macrogen Inc, Korea.

2.2.3 Sequence analysis.

The full length BFDV genomes were assembled using DNAMAN (version 5.2.9; Lynnon Biosoft), and the genomes were aligned to all other BFDV genomes available in GenBank using the ClustalW-based sub-alignment tool available in MEGA5 (gap open penalty = 10; gap extension penalty = 5) (He *et al.*, 2007); with manual editing. Maximum-likelihood phylogenies were inferred using PHYML (Guindon and Gascuel, 2003), with 1000 non-parametric bootstrap replicates and the model GTR + G4 (determined using MODELTEST) (Horlen *et al.*, 2008). Branches with less than 50% support were collapsed using Mesquite (Version 2.75). Recombination was analysed using RDP4 (Martin *et al.*, 2010), using the RDP (Martin and Rybicki, 2000), GENECONV (Padidam *et al.*, 1999), BOOTSCAN (Martin *et al.*, 2005), MAXCHI (Smith, 1992), CHIMAERA (Posada and Crandall, 2001), SISCAN (Gibbs *et al.*, 2000), and 3SEQ (Boni *et al.*, 2007) methods. Recombination events were judged to be credible if they were detected by more than three methods with significant p-values, combined with phylogenetic evidence of recombination.

2.3 Results

2.3.1 Screening of New Zealand parrots for BFDV.

Of the 753 native birds sampled, 16 Red-fronted Parakeets from Little Barrier Island tested positive for BFDV, suggesting a prevalence of infection of 10.5% (16/153; 95% CI: 6.1%-16.4%). Following

reports of feather loss in wild populations of Yellow-crowned Parakeets in the Eglinton region of the South Island, 30 birds were sampled between November 2011 and February 2012. Of these, eight tested positive for BFDV, indicating a prevalence of 26.7% (8/30; 95% CI: 12.3%-45.9%). From the exotic Eastern Rosellas sampled, seven birds in the Auckland region tested positive, with a prevalence of 21.9% (7/32; 95% CI: 9.9%-42.3%).

2.3.2 Full genome sequence analysis of BFDV isolates.

Full genome analysis of the New Zealand BFDV isolates shows that the Red-fronted Parakeet isolates share >99% pairwise sequence identity, while the Yellow-crowned Parakeet isolates share >98% sequence identity. Eastern Rosella isolates share between 93.8%-97.7% identity. Pairwise sequence identity between isolates from the Red-fronted Parakeets in the North and Yellow-crowned Parakeets in the South ranges from 92.7%-93.4%, and these results, when combined with the maximum-likelihood phylogenetic analysis, show that the origins of the BFDV infection in these two populations is different (Fig. 2.2). Isolates from the Red-fronted Parakeets and the Eastern Rosellas share a common ancestor, as seen in the ML phylogenetic analysis, and from the 94.9%-97.9% pairwise sequence identity between these isolates. These isolates form a monophyletic clade belonging to the BFDV-A strain. Isolates from the Yellow-crowned Parakeets, however, share only 92%-94% sequence identity with the Eastern Rosella isolates, and 84%-94% sequence identity with all full genome BFDV isolates available on GenBank, including isolates from this study. They are sufficiently different to be designated a new strain, BFDV-O, and are most closely related to BFDV-I isolates from South Africa and Europe (Fig. 2.2). Maximum-likelihood analyses of partial *rep* gene sequences (574 bp) and *cp* gene sequences (404 bp) of the New Zealand isolates and all others deposited in GenBank (*rep*: n=162; *cp*: n=191) supported these results (Fig. 2.3 and 2.4).

2.3.3 Recombination analysis.

Recombination was detected in two Eastern Rosella isolates, representing genotypes BFDV-A_5 and BFDV-A_6 (Fig. 2.5). Both isolates had a recombinant region of unknown origin detected in the C-terminal portion of the *rep* gene, and BFDV-A_6 had an additional recombinant region in the *cp* gene from a Yellow-crowned Parakeet BFDV-O isolate.

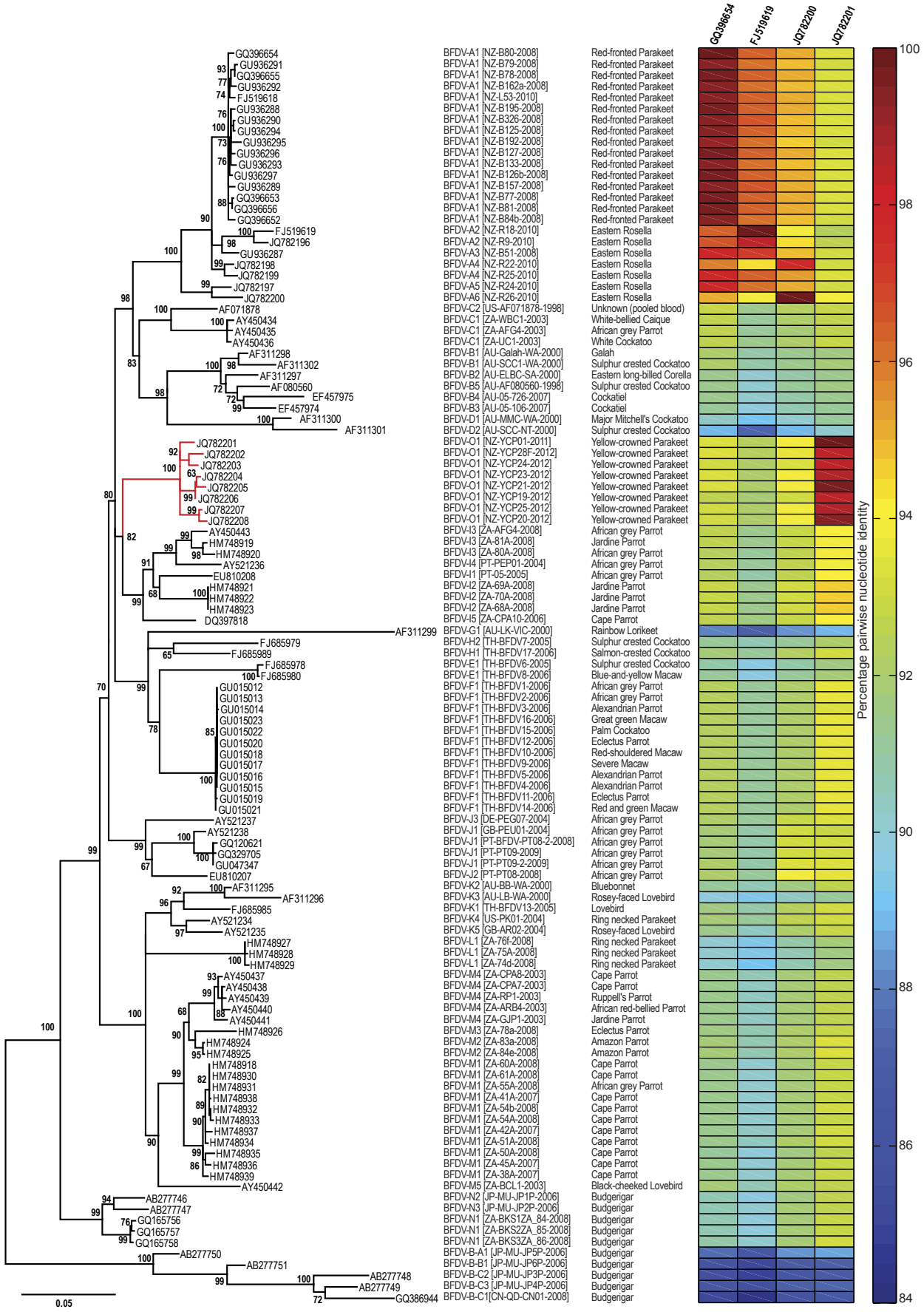


Fig. 2.2. Left: Maximum-likelihood phylogenetic tree, constructed in PHYML using the model GTR+G4 with 1000 full maximum-likelihood bootstrap replicates, showing the relationships of the full genome sequences of the New Zealand BFDV isolates to all other publically available full-length BFDV genomes. Right: Pairwise nucleotide identities of four NZ isolates, representing strains BFDV-A_1 (GQ396654), -A_2 (FJ519619), -A_6 (JQ782200), and BFDV-O_1 (JQ782201), to all other isolates.

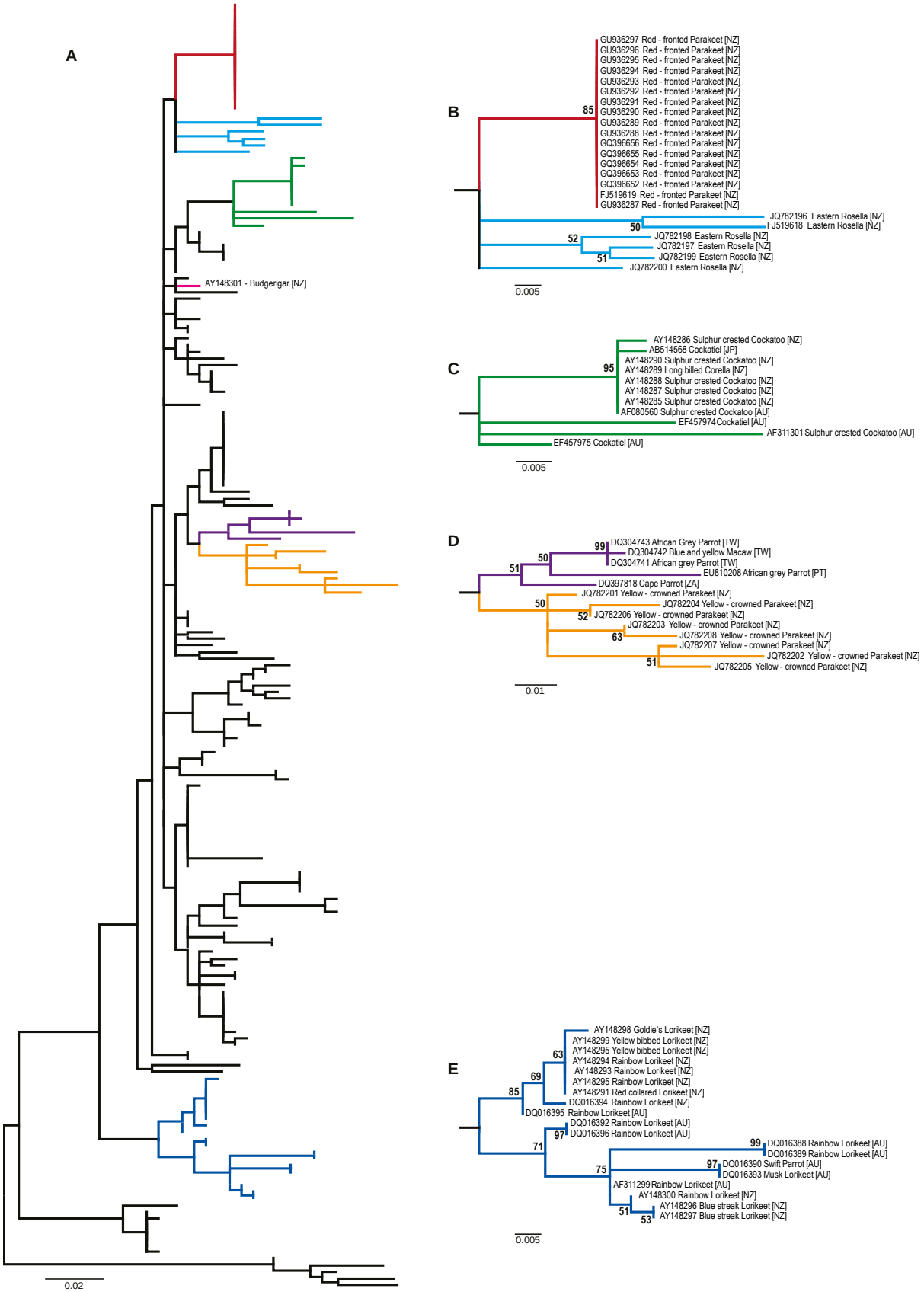
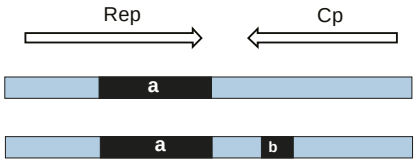


Fig. 2.3. A. Maximum-likelihood phylogenetic tree constructed in PHYML, showing the relationships of partial (~ 574 nt) Rep sequences of all partial and full BFDV genomes available in GenBank. **B**, **C**, **D**, and **E** indicate the clades with New Zealand isolates.

Fig 2.4. A. Maximum-likelihood phylogenetic tree constructed in PHYML using model GTR+G4 showing the relationships between partial (~404 nt) BFDV CP sequences in GenBank. **B.** NZ Yellow-crowned Parakeet sequences. **C.** NZ Eastern Rosella and Red-fronted Parakeet sequences.



Event	Recombinant	Recombinant region	Potential major parents	Potential minor parent	Detection method	P-value
a	BFDV-A_3-[NZ-R24-2010]	428-1000	BFDV-A	Unknown	RGBM**S**T	3.3×10^{-15}
b	BFDV-A_6-[NZ-R26-2010]	1225-1384	BFDV -A_4, -A_5	BFDV-O	RGBM**S**T	3.9×10^{-5}

Fig. 2.5. Cartoon illustration and details of recombination events detected in New Zealand BFDV isolates using RDP4 and the methods RDP (R), GENECONV (G), BOOTSCAN (B), MAXCHI (M), CHIMAERA (C), SISCAN (S), and 3SEQ (T). The detection method was only included in the table if it was significant. P-value is from the detection method indicated in bold.

2.4 Discussion

This study provided the first evidence of BFDV in the South Island of New Zealand, and reconfirmed the presence of BFDV in Red-fronted Parakeets and Eastern Rosellas in the North Island. Isolates from the North Island fall under the same strain, BFDV-A, whereas the Yellow-crowned Parakeet isolates from the South Island form a new strain entirely, designated BFDV-O. The low level of divergence among isolates from the Red-fronted Parakeet population and the Yellow-crowned Parakeet population indicates a relatively recent infection. Despite the Red-fronted Parakeets and Eastern Rosellas being infected with a closely related strain, the greater degree of genetic diversity within the Eastern Rosella isolates (reflected by the different subtypes) indicates that they have been infected for a longer period of time, thus it is possible that they transmitted the virus to the Red-fronted Parakeets. Both BFDV-A and BFDV-O have only ever been sampled in New Zealand, therefore it could be possible that the Eastern Rosellas became infected within New Zealand, and evidence of recombination in two Eastern Rosella isolates with an unknown strain indicates that there are more strains in New Zealand that have yet to be sampled.

The discovery of BFDV within two native New Zealand species, the Yellow-crowned Parakeet and the Red-fronted Parakeet, and the fact that two different strains are present, raises concerns for other native psittacine species, particularly those that are classed as at greater risk of extinction, such as the Kakapo. While BFDV has so far not been detected within the Kakapo population, the decision to translocate individuals to Little Barrier Island, where BFDV has been clearly demonstrated, must surely be questioned, as exposure to this potentially fatal virus will likely have serious implications for the ongoing management and recovery of this critically endangered species.

No birds within captive facilities were found to be positive for BFDV in this study, but the captive environment is one that facilitates transmission of BFDV. Birds under stress have lowered immunity,

so are more likely to become infected, and the close proximity that birds are kept in enables recombination of isolates, with the potential emergence of novel virus strains. The use of these captive-bred birds as founder stock for wild populations, or to supplement the same, means that novel strains of BFDV could be transferred into new environments, putting other species at risk. Thus, a comprehensive screening programme is an absolute must for any birds that are released from captivity, brought into captivity, or translocated from one region to another.

Full genome analysis, including recombination analysis, is vital for determining the evolutionary history of BFDV isolates, and has the potential to be used to track the source of an infection. In this study, it enabled the detection and molecular characterisation of a novel strain of BFDV in Yellow-crowned Parakeets. With extensive continued sampling and full genome analysis of isolates, it may eventually be possible to determine the source of the original strain to have entered New Zealand.

Chapter three: First report of BFDV in New Caledonia, and Evidence of Multiple Introductions

Abstract

Psittacine beak and feather disease (PBFD), caused by *Beak and feather disease virus* (BFDV), is a potentially fatal disease of psittacines. The almost global presence of BFDV has serious implications for the future of psittacine species, over a quarter of which are considered to be at risk of extinction. This study documents the first discovery of BFDV in the Pacific islands of New Caledonia, home to three at risk parrot species. One hundred and sixty eight exotic and 79 endemic birds were sampled, of which 26 tested positive for BFDV. Complete genomes of the New Caledonian BFDV isolates were characterised, and phylogenetic analysis revealed a completely new viral strain, BFDV-P_1, isolated from 17 Deplanche's Rainbow Lorikeet (*Trichoglossus haematodus deplanchii*), an endemic subspecies of the Rainbow Lorikeet (*T. haematodus*). The remaining nine isolates, including one from a vulnerable, endemic New Caledonian Parakeet (*Cyanoramphus saisseti*), were placed into an existing viral strain, BFDV-J_1. This study indicates that there have been at least two separate introductions of BFDV into New Caledonia, and highlights the need for comprehensive screening of birds before they are transported as part of the international pet trade, in order to prevent introductions into BFDV-naïve areas. Regular surveillance of both wild and captive populations is important, so that risk management and biosecurity protocols can be developed to prevent further introductions of BFDV and minimise the spread of the existing viral strains.

3.1 Introduction

The French territory of New Caledonia lies in the south-west Pacific Ocean, approximately 1200km to the east of Australia and 1700km to the north of New Zealand. The territory is comprised of the main island, Grand Terre, and a number of smaller islands. New Caledonia is home to three endemic psittacine species: the Horned Parakeet (*Eunymphicus cornutus*), found on the mainland Grand Terre; the Ouvéa Parakeet (*Eunymphicus uvaeensis*), found only on the island of Ouvéa, part of the Loyalty Island archipelago some 100km north-east of the mainland; and the New Caledonian Parakeet (*Cyanoramphus saisseti*), also found on the mainland. Once thought to be a subspecies of the New Zealand Red-fronted Parakeet (*C. novaezelandiae*), the New Caledonian Parakeet is now recognised as a species in its own right, basal to other *Cyanoramphus* species (Boon *et al.*, 2001). All three species have been classed as at risk of extinction in the wild; the Horned Parakeet and New Caledonian Parakeet are listed as vulnerable, while the Ouvéa Parakeet, with a much more restricted range, is classed as endangered (BirdLife International, 2012), despite an increase of numbers in the wild due to the cessation of nest poaching for the pet trade (Barré *et al.*, 2010). A fourth psittacine species present in New Caledonia is a subspecies of the Rainbow Lorikeet (*Trichoglossus haematodus*). Up to 20 subspecies of Rainbow Lorikeet are recognised, based on differences in colouration, and are widespread throughout northern and eastern Australia, Indonesia and many of the Pacific islands (Forshaw, 2010). The Deplanche's Rainbow Lorikeet (*T. h. deplanchii*), occasionally referred to as the New Caledonian Rainbow Lorikeet, is one such subspecies found only in New Caledonia, predominantly on the mainland of Grand Terre (Barré *et al.*, 2010; Legault *et al.*, 2011). The Deplanche's Rainbow Lorikeet is not considered to be at risk of extinction.

Along with the endemic psittacine species in New Caledonia, many different exotic species have been imported over the years, for public display in zoos or for breeding in public and private aviaries. Many of these captive facilities also house endemic New Caledonian species, for conservation education and breeding purposes. With the importation of exotic psittacine species comes the risk of also importing BFDV, and housing exotic psittacines in the same facilities as endemic species increases the risk of transmission of the virus to the threatened native species, yet there have been no previous reports of BFDV in New Caledonia. Recently, however, a deceased wild Deplanche's Lorikeet was discovered in a park in the South Province of New Caledonia, and no obvious cause of death could be identified. Once further investigation revealed birds suffering from feather loss in free-living populations of Deplanche's Lorikeet, the possibility of an outbreak of BFDV could not be ignored. Thus large scale sampling of both exotic and endemic species was undertaken, with most samples coming from captive aviaries. Samples were also obtained from 17 Deplanche's Lorikeets

that had been rescued from the wild suffering from feather loss, and were being nursed in captivity. In all, 247 birds were sampled from New Caledonia, encompassing 25 species in 19 genera. Along with the New Caledonian samples, a feather sample from a symptomatic, deceased Rainbow Lorikeet (*T. haematodous*) nestling from Australia provided by Dr David Collings from the University of Canterbury was also analysed.

3.2 Materials and methods

3.2.1 Sampling, DNA extraction, and PCR screening for BFDV.

Blood and/or feather samples were collected from 247 birds in New Caledonia by Almudena Lorenzo, Jean-Paul Chenuet, Marianne Bonzon, Celine Marchal, Laurent Vignon, Bethany Jackson, and Arvind Varsani and sent to the Virology Laboratory at the University of Canterbury, to be tested for BFDV. Of the 247 birds, 39 were native at risk species (*C. saisseti*, n=14; *E. cornutus*, n=17; *E. uvaeensis*, n=8), 40 were the endemic Rainbow Lorikeet subspecies Deplanche's Lorikeet, and the remaining 168 were exotic species, although all had been hatched and fledged in New Caledonia (Table 3.1). Blood was collected on filter paper by venipuncture of the medial metatarsal vein in the leg. Either 2mm of the calamus of the feather was excised using a sterile scalpel and placed into a sterile 1.5ml microcentrifuge tube, or the dried blood spot was excised from the filter paper with a sterile scalpel, cut into smaller pieces, and placed into a sterile 1.5ml microcentrifuge tube. Total DNA from the feather and blood samples was then extracted using the Genomic DNA extraction kit for blood (Intron Biotechnology, Korea), according to the manufacturer's instructions.

Four microlitres of total extracted DNA was then used for PCR based screening using primers targeting the ~605bp region of the Rep gene (5'-TTA ACA ACC CTA CAG ACG GCG A and 5'-GGC GGA GCA TCT CGC AAT AAG) (Ritchie *et al.*, 2003). The PCR protocol was as previously noted (see section 2.2.1), and PCR products were resolved on a 1% agarose gel stained with SYBR® Safe DNA stain.

3.2.2 Amplification and cloning of full BFDV genomes

Samples that tested positive for BFDV through the PCR-based screening method were subjected to rolling circle amplification (RCA) using TempliPhi™ (GE Healthcare, USA), as described in section 2.2.2, to amplify the full viral genome. After 20 hours, the resulting linear concatemers were used as enriched viral targets for a PCR based full genome amplification using back-to-back primers (target in the conserved domain of Rep; AV-BFDV-F 5-CYT ACY CTK GGC ATT GTG GC-3', AV-BFDV-R 5'-TAT HAC

RTC BCC YTC YTT ACT GCA-5'; primers were designed based on the conserved region of Rep from full BFDV genomes available in GenBank) using Kapa HiFi HotStart DNA polymerase (Kapa Biosystems, USA) with 1µl of the TempliPhi™ product and the following touchdown PCR protocol: initial denaturation of 94°C for 2 min; 10 cycles of 98°C for 20s, 62°C for 20s, and 72°C for 2 min; 20 cycles of 98°C for 20s, 52°C for 20s, and 72°C for 2 min; and a final extension of 72°C for 2 min. The resulting amplicons were separated on a 0.7% agarose gel and the ~2 kb bands excised, recovered using Mega-spin agarose gel extraction kit (Intron Biotechnology, Korea) and ligated into pJET1.2 vector (CloneJET™ PCR cloning kit, Fermentas, USA). Plasmid from a transformed *E.coli* colony was isolated and sequenced by primer walking at Macrogen Inc. (Korea).

Table 3.1. List of species tested for BFDV in New Caledonia. Also analysed, but not included in the table, was a feather from an Australian Rainbow Lorikeet (*Trichoglossus haematodus*).

Scientific name	Common name	Number tested	Number positive
Agapornis fischeri	Fischer's Lovebird	2	0
Agapornis roseicollis	Peach-faced Lovebird	6	0
Alisterus scapularis	Australian King Parrot	4	0
Amazona barbadensis	Yellow -shouldered Amazon	38	0
Ara chloropterus	Red-and-green Macaw	6	0
Ara macao	Scarlet Macaw	2	0
Ara militaris mexicana	Mexican Military Macaw	4	0
Ara militaris x macao	Military/Scarlet Macaw hybrid	2	0
Aratinga solstitialis	Sun Conure	8	0
Cacatua galerita galerita	Sulphur-crested Cockatoo	2	0
Cacatua sanguinea	Little Corella	2	0
Cyanoramphus saisseti	New Caledonian Parakeet	14	1
Eclectus roratus vosmaeri	Eclectus Parrot	13	6
Eolophus roseicapilla	Galah	9	0
Eunymphicus cornutus	Horned (crested) Parakeet	17	0
Eunymphicus uvaeensis	Ouvea Parakeet	8	0
Lorius chlorocercus	Yellow-bibbed Lory	2	0
Melopsittacus undulatus	Budgerigar	14	0
Nymphicus hollandicus	Cockatiel	11	0
Platycercus eximius	Eastern Rosella	2	0
Poicephalus senegalus	Senegal Parrot	25	0
Psephotus haematonotus	Red-rumped Parrot	1	1
Psittacula krameri	Ring-necked Parakeet	9	1
Psittacus erithacus	African Grey Parrot	6	0
Trichoglossus haematodus deplanchii	Deplanche's Rainbow Lorikeet	40	17
Total		**247**	**26**

3.2.3 Bioinformatic analysis

The BFDV contigs were assembled into full length genome sequences using DNAMAN (version 5.2.9; Lynnon Biosoft). Assembled genomes were aligned with all others available in GenBank, including the recently characterised New Zealand isolates (chapter two), using ClustalW (gap open penalty = 10; gap extension penalty = 5) with manual editing done using MEGA 5 (Tamura *et al.*, 2011). PHYML (Guindon and Gascuel, 2003) was used to infer maximum likelihood (ML) phylogenies, with 1000 non-parametric bootstrap replicates and model GTR+I+G4 as determined by RDP4 (Martin *et al.*, 2010). ML trees of the amino acid sequences of the Rep and CP were constructed using the LG model in PHYML (Guindon and Gascuel, 2003), with aLRT branch support (Anisimova and Gascuel, 2006). Branches that had less than 60% support were collapsed using Mesquite (version 2.75), and pairwise comparison (p-distance with pairwise deletion of gaps) of the full BFDV genomes, the Rep residues and the CP residues were performed using MEGA 5 (Tamura *et al.*, 2011). Recombination among the New Caledonian BFDV isolates was analysed using RDP4 (Martin *et al.*, 2010) with default setting and the following methods implemented: RDP (Martin and Rybicki, 2000), GENECONV (Padidam *et al.*, 1999), BOOTSCAN (Martin *et al.*, 2005), MAXCHI (Smith, 1992), CHIMAERA (Posada and Crandall, 2001), SISCAN (Gibbs *et al.*, 2000), and 3SEQ (Boni *et al.*, 2007). Only recombination events that were detected by more than three of these methods with significant p-values and phylogenetic support were considered to be credible events.

3.3 Results and discussion

3.3.1 PCR screening for BFDV

Of the 247 birds tested, 26 were positive for BFDV, the majority of which were the Deplanche's Rainbow Lorikeet (17/40, prevalence = 42.5%, 95% CI: 27-59.1%), all of which had displayed classic symptoms of BFDV infection, including generalised feather loss and failure to grow primary feathers. Eight of the other positive results were from exotic species: six Eclectus Parrots (*Eclectus roratus vosmaeri*; 6/10, prevalence = 60%, 95% CI: 26.2-87.8%) and single specimens of the Red-rumped Parrot (*Psephotus haematonotus*; 1/1, prevalence = 100%, 95% CI: 2.5-100%) and the Ring-necked Parakeet *(Psittacula krameri*; 1/9, prevalence = 11.1%, 95% CI: 0.3-48.2%). Of concern was the positive result from the New Caledonian Parakeet (*Cyanoramphus saisseti*), a species already considered vulnerable, with a single bird found to be infected (1/14, prevalence = 7.14%, 95% CI:

0.2-33.9%). The feather sample from the Australian Rainbow Lorikeet (*Trichoglossus haematodus*) also tested positive for BFDV.

3.3.2 Full genome sequence analysis of BFDV isolates.

Within all the genomes we identified: 1) the circovirus nonanucleotide origin of replication sequence (TAGTATTAC); 2) three conserved rolling circle replication motifs (FTLNN, GxxHLQGY, YxxK) in the Rep; 3) the nuclear localisation motif (RRR x ARPY x RRRH x RR x R xx RRRR x FRRRRFST x RIYTLRL x RQ) on the N-terminus of the CP.

Full genome analysis showed that the New Caledonian isolates fall into two strains, BFDV-J_1 (n=9), and a new strain, BFDV-P_1 (n=17), based on the classification system proposed by Varsani *et al.* (2011). The Australian isolate is a subtype of the BFDV-G strain, BFDV-G_2, and shares ~95% sequence identity from a Rainbow Lorikeet sequence previously isolated in Australia, BFDV-G_1 (Fig. 3.1).

The nine BFDV-J_1 isolates from Eclectus Parrots (n=6), a New Caledonian Parakeet (n=1), a Red-rumped Parrot (n=1) and a Ring-necked Parakeet (n=1) share >98% pairwise sequence identity amongst themselves, and a similar degree of identity to a BFDV-J_1 isolate from an infected African Grey Parrot (*Psittacus erithacus*) from Portugal, while sharing ~95% identity to other European isolates from African Grey Parrots (UK, Portugal, and Germany; Fig. 3.1).

The remaining 17 isolates, all from Deplanche's Rainbow Lorikeets, form a new strain, BFDV-P_1. These isolates are highly similar to each other, sharing >97% genome wide pairwise identity. However they share only ~90% pairwise identity with the Australian Rainbow Lorikeet isolates BFDV-G_1 and -G_2 (Fig. 3.1). All of the Rainbow Lorikeet isolates (BFDV-P and -G) are distinct from all other BFDV isolates, and according to maximum likelihood phylogenetic analysis, they share a common ancestor. Thus the New Caledonian BFDV-P strain among the Deplanche's Rainbow Lorikeets has probably originated from the Australian Rainbow Lorikeets, isolates of which, with ~90% pairwise identity, are most closely related to BFDV-E, -F and -H isolates from Thailand (Fig. 3.1).

3.3.3 Rep and CP sequence analysis.

The New Caledonian BFDV-P_1 Rep sequences share >98.6% pairwise identity amongst each other, and 91% identity to the Reps from Australian Rainbow Lorikeets, which share 97.6% identity. The Reps from New Caledonian BFDV-J_1 isolates share >99% identity amongst themselves and to European BFDV-J_1 isolates, as well as to BFDV-K_1 from Thailand. Maximum likelihood analysis based on the Rep sequences places the New Caledonian and European BFDV-J_1 isolates together with

BFDV-K_1 from Thailand, and BFDV-K_2 and -K_3, both from Australia (Fig. 3.2). Amongst themselves, all the Rainbow Lorikeet isolates share ~91.3–92.7% pairwise identity.

The CPs of the Australian Rainbow Lorikeet isolates proved to be divergent, sharing only 88.9% pairwise identity to each other, and 86.4-91.8% identity to BFDV-P_1 CPs, which share >96.3% identity amongst each other. All of the Rainbow Lorikeet isolates, BFDV-P_1, BFDV-G_1 and -G_2, share ~85-87% identity in the CP, and the BFDV-J_1 CPs share 95.1% identity (Fig. 3.2). A comparison of the Rep and CP phylogenetic trees shows differences in the clustering of isolates, which is possibly attributable to recombination. For example, BFDV-K isolates cluster with BFDV-J isolates in the Rep tree, but with BFDV-L isolates in the CP maximum likelihood phylogenetic tree.

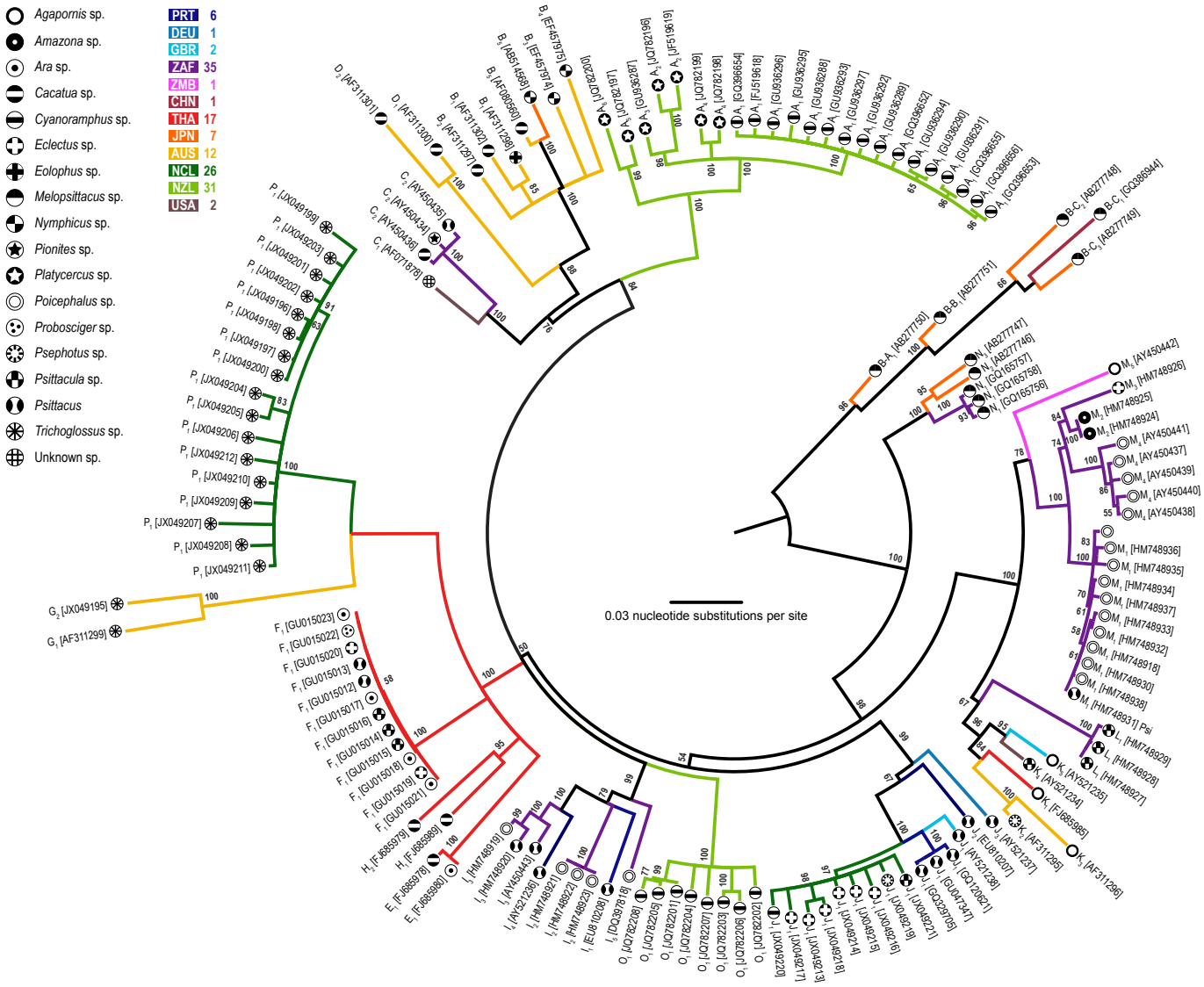


Fig. 3.1. Maximum-likelihood phylogenetic tree showing the relationships of the full genome sequences of the New Caledonian BFDV isolates to all other available BFDV full genome sequences in GenBank. The tree was constructed in PHYML using model GTR+I+G4, with 1000 non-parametric bootstrap replicates. Colours represent the isolate country of origin, with New Caledonia shown in dark green. Symbols represent the species infected.

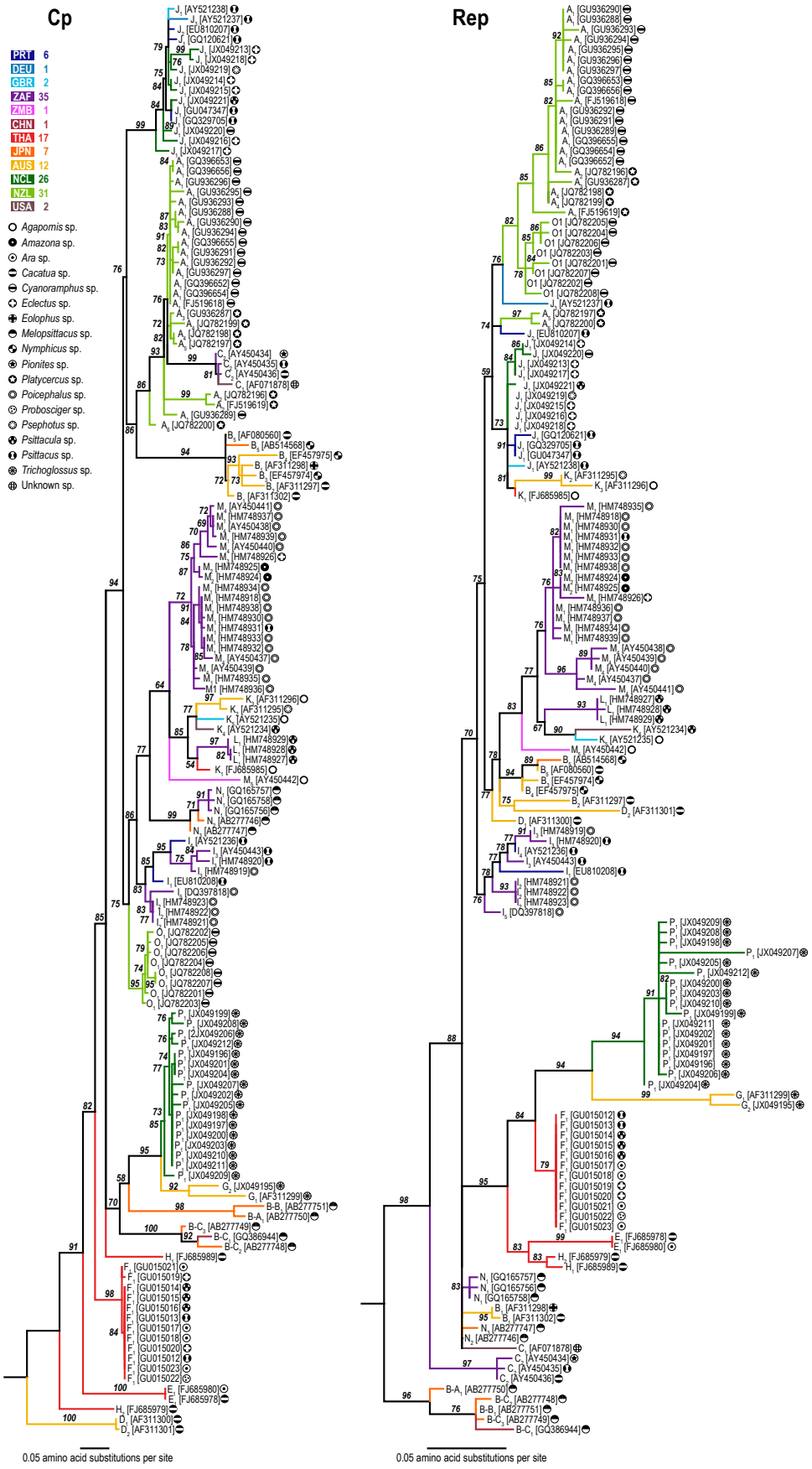


Fig. 3.2. Maximum-likelihood phylogenetic tree of the amino acid sequences of Rep and CP from BFDV isolates. The trees were constructed in PHYML using the LG model, with aLRT branch support. New Caledonia isolates are again in dark green.

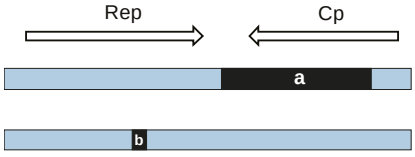
The calculated ratios of normalised synonymous (dS) and non-synonymous (dN) substitution rates from codon alignments of CP and Rep genes using SLAC method (Pond and Frost, 2005; Pond *et al.*, 2006) implemented in the online server DATAMONKEY, at http://www.datamonkey.org/ (Delport *et al.*, 2010), show that both Rep (dN/dN = 0.189574) and CP (dN/dN = 0.353265) are evolving under purifying selection (dN/dS < 1). Within the CP (249 codons) we note 0.752754 substitutions per site, whereas in the Rep (291 codons) it is 0.490136 substitutions per site, providing clear evidence that the Rep genes are evolving under a greater degree of negative selection compared to the CP genes.

Therefore, not only do the Rep and CP phylogenetic trees show variation in the clustering of isolates (Fig. 3.2), but they demonstrate that the Rep gene is more conserved than the CP gene, which can be put down to intraspecies antigenic variation. These differences highlight the fact that phylogenetic analyses must be based on complete viral genomes, in order to properly classify BFDV isolates, and comparisons between phylogenies cannot be made purely based on either the *Rep* or *CP* genes.

3.3.4 Recombination analysis.

All the BFDV-J_1 isolates were recombinants, with an approximately 700bp fragment from Australian BFDV-K isolates (Fig 3.3). The region of recombination spanned the C-terminal portion of the *cp* gene and the intergenic region, which have previously been shown to be recombination hotspots (Heath *et al.*, 2004; Lefeuvre *et al.*, 2009; Varsani *et al.*, 2011). This would indicate that the New Caledonian BFDV-J_1 isolates probably originated from Europe, perhaps entering the country when infected parrots were imported from European breeding facilities.

Another recombination event was detected in a single BFDV-P_1 isolate, from a Deplanche's Rainbow Lorikeet, which has an approximately 70bp recombinant region within the *rep*, from a BFDV variant that has yet to be sampled (Fig 3.3). This indicates that the BFDV diversity in New Caledonia may be greater than has been detected in this study.



Event	Recombinant	Recombinant region	Potential major parents	Potential minor parent	Detection method	P-value
a	BFDV-J_1	1218-1929	BFDV-J_2	BFDV-K, -M, -L	RGMC**S**T	1.1×10^{-13}
b	BFDV-P_1-[NC-2NC40F-2011]	635-706	BFDV -P_1	Unknown	R**G**B	5.1×10^{-6}

Fig. 3.3. Cartoon illustration and details of recombination events detected in New Caledonian BFDV isolates, using RDP4 and the methods RDP (R), GENECONV (G), BOOTSCAN (B), MAXCHI (M), CHIMAERA (C), SISCAN (S), and 3SEQ (T). The detection method was only included in the table if it had a significant result. The p-value is for the method shown in bold.

3.4 Concluding remarks

This study shows that there have been at least two introductions of BFDV into New Caledonia. Isolates from captive birds, three exotic species (Eclectus Parrot, n=6; Red-rumped Parrot, n=1; and Ring-necked Parakeet, n=1) and one endemic species (New Caledonian Parakeet, n=1) were all from the already sampled BFDV-J_1 strain, and were most similar to BFDV-J isolates from Europe, indicating that the New Caledonian infection probably arose from the importation of an infected bird from Europe. Isolates from the wild parrots, Deplanche's Rainbow Lorikeet (n=17), formed an entirely new strain, BFDV-P_1, and were most similar to Australian Rainbow Lorikeet BFDV-G isolates. The Rainbow Lorikeet isolates as a group are distinct from all other BFDV isolates and share a common ancestor, suggesting a lorikeet specific lineage, as has been previously noted (Khalesi *et al.*, 2005; Ritchie *et al.*, 2003).

The presence of BFDV in the wild in New Caledonia raises serious concerns for the at risk endemic species. This study has shown that the New Caledonian Parakeet is susceptible to infection, and the *Eunymphicus* genus has previously been reported to be susceptible (Tomasek and Tukac, 2007). Although BFDV-P_1 so far appears to be lorikeet specific, the observed recombination with an unknown BFDV variant (Fig. 3.3) not only suggests that more strains have yet to be sampled in New Caledonia, but raises the possibility that recombination of strains could result in the emergence of a new viral strain with an increased host range and perhaps greater virulence. New Caledonian Parakeets, Horned Parakeets, and Deplanche's Rainbow Lorikeet all inhabit the mainland of New Caledonia, Grand Terre, which could increase the chances of transmission from the lorikeets to the parakeets, although the apparant preference of parakeets for higher altitudes than the lorikeets may reduce the chances of encountering each other in the wild (Legault *et al.*, 2011). The Ouvéa Parakeet is found only on the island of Ouvéa (area ~130km^2), and although the Deplanche's Lorikeet was implicated as a threat to the survival of the parakeet through competition for nest sites, a 16 year study monitoring the parakeet reported that sightings of the lorikeet were very rare, and that it was never observed in the habitat used by the parakeet (Barré *et al.*, 2010). Thus, the relative isolation of the Ouvéa Parakeet may actually work to protect it from BFDV infection in the wild.

That captive birds were found to be infected is of no surprise, as the captive environment is one that facilitates transmission of BFDV. The environmental stability of the virus and the inherent difficulties in completely disinfecting an aviary mean that BFDV can be present and transmissible for a long period of time. The BFDV infection in captive birds in New Caledonia is a classic example of cross-species transmission within a captive environment, as multiple species are infected with the same strain, BFDV-J_1, with very little divergence among isolates (isolates share >98% pairwise sequence

identity). This sort of situation is exactly why a comprehensive testing regime should be adopted worldwide, so that any parrot that is moved between countries is BFDV free. Additional screening should be performed before any imported parrot is allowed to join an existing aviary.

Full genome sequence analyses reveal a great deal about the evolutionary history of BFDV, and can also be useful in tracking the source of an infection. In this study, the phylogenetic analysis of complete BFDV genomes, along with recombination analysis, enabled the identification of a new strain of BFDV, BFDV-P_1. However, it also allowed the tracking of a second strain, BFDV-J_1, to its likely European origin. Continued sampling of parrots in New Caledonia is needed, to determine if there are more strains present than this study identified. In particular, testing of wild populations of the three endangered psittacine species would be beneficial to determine the current BFDV status, which can help inform any future conservation management decisions.

This chapter has been published: **Julian, L.**, Lorenzo, A., Chenuet, J-P., Bonzon, M., Marchal, C., Vignon, L., Walters, M., Collings, D.A., Jackson, B. and Varsani, A. (2012) Evidence of multiple introductions of Beak and feather disease virus into the pacific islands of Nouvelle-Calédonie (New Caledonia) Journal of General Virology 93: 2466 – 2472

Chapter four: Genetic Diversity of BFDV isolates in Poland

Abstract

Of the seven continents, Europe is the only one hospitable to life that is not home to any native parrot species. However, after the first introduction in 327BC, Europe has been one of the major importers of wild caught exotic parrots. The banning of this practice has seen the demand for parrots for the international pet trade met by established European breeding facilities. However, years of unregulated global trade in exotic psittacines means it is likely that birds within breeding facilities are infected with BFDV. In this study, 209 birds were tested from over 50 captive facilities across Poland, a European country previously shown to have BFDV. Forty three birds tested positive, from 14 species within 10 genera, with BFDV cases spread out among 18 different facilities. The genomes of all the Polish BFDV isolates were isolated and characterised, and phylogenetic analysis revealed six new strains of BFDV, along with four new subtypes of existing strains. The recombination analysis demonstrated that the Polish BFDV isolates were highly recombinant, with most strains showing evidence of recombination events. It is clearly evident that there have been multiple introductions of BFDV into Poland over a long period of time, and as these birds are kept in captivity, the ideal environment has arisen for evolution of novel strains of BFDV through recombination. The results of this study emphasise the need for comprehensive screening programs to ensure freedom from disease before any bird is allowed to join an established flock, whether that be a wild or captive flock, and thus minimise the risk of evolution of new strains of BFDV with altered pathogenicity.

4.1 Introduction

The natural habitat of wild parrots is tropical to subtropical regions, predominantly in the Southern hemisphere (Forshaw, 2010), although they are also naturally found in more temperate regions such as the islands of New Zealand. Of the seven continents, only Antarctica and Europe have no native parrots. However their desirability as pets, in particular for the aristocracy, means that Europe has historically been one of the major importers of wild-caught exotic parrots. The first introduction of exotic parrots occurred when Alexander the Great returned home from India in 327BC, bringing with him a parrot that was subsequently named the Alexandrine Parakeet (*Psittacula eupatria*) (Boehrer, 2004). The practice of importing wild-caught parrots was prohibited in 2007 (Commission Regulation (EC) No. 318/2007), although naturally, this legislation does not prevent illegal trafficking of psittacine species. As long as the trade in parrots was unregulated, many different species of parrots were imported for the pet trade or for personal collection, and the occasional accidental or deliberate release resulted in some species establishing populations in the wild. As such, psittacine species now account for approximately 18% of Europe's established wild populations of introduced birds (Strubbe and Matthysen, 2009). The legislation prohibiting importation of wild-caught exotic parrots did not reduce the demand for pet parrots, and as such that demand is currently met by established and registered breeders within Europe, who are also able to export birds to countries or buyers outside Europe. While these facilities may have been advantageous in assisting with the conservation of some endangered psittacine species, these captive facilities can also provide an ideal environment for cross-species transmission of BFDV, and rapid evolution of new strains through recombination.

Previously, BFDV infection has been reported in various captive psittacine breeding facilities in Europe (Bert *et al.*, 2005; Henriques *et al.*, 2010; Piasecki and Wieliczko, 2010; Rahaus and Wolff, 2003; Tomasek and Tukac, 2007), however, very few full genome analyses have been performed to accurately infer phylogenetic relationships, source of the BFDV infections and to detect recombination events between strains. In this study, full viral genomes isolated from BFDV positive birds from across Poland were analysed in an attempt to gain a greater insight into the diversity of BFDV, generally circulating strains of BFDV, and to assess the recombinant nature of these viruses in Europe. Our results show that there have been multiple introductions of BFDV into Poland, and that the strains are highly recombinant.

4.2 Materials and methods

4.2.1 Sampling, DNA extraction, and PCR screening for BFDV.

Blood and/or feather samples were obtained from 209 birds encompassing 23 genera from more than 50 captive facilities in Poland (Table 4.1). The samples were pre-processed by Drs Tomasz Piaseki and Klaudia Chrzastek and total DNA from samples was sent to the virology lab at UC for further analysis as part of a collaborative study. In brief, DNA was extracted from the blood or feather sample of birds using iGenomic blood DNA extraction kit (Intron Biotechnology, Korea) according to the manufacturer's instructions. Four microlitres of total extracted DNA was then used for PCR based screening using KAPA Blood PCR Kit Mix B (KAPA Biosystems, South Africa) and primers targeting the ~605bp region of the Rep gene (5'-TTA ACA ACC CTA CAG ACG GCG A-3' and 5'-GGC GGA GCA TCT CGC AAT AAG-3') (Ritchie *et al.*, 2003). The PCR protocol was as previously noted (see section 2.2.1), and PCR products were resolved on a 1% agarose gel stained with SYBR® Safe DNA stain.

4.2.2 Rolling circle amplification (RCA) and isolation of full BFDV genomes

Samples that tested positive for BFDV using the screening method described above were subjected to RCA using TempliPhi™ (GE Healthcare, USA) as previously described (see section 2.2.2) to amplify the full viral genome. The resulting linear concatemers were used in two different approaches to isolate the full BFDV genome. In the first, 1µl of the amplified TempliPhi™ product was digested with the restriction enzyme *Bam*HI. In the second approach, 1µl of the amplified TempliPhi™ product was used in a second PCR reaction to amplify the full BFDV genome using back-to-back primers (target in the conserved domain of Rep; AV-BFDV-F 5'-CYT ACY CTK GGC ATT GTG GC-3', AV-BFDV-R 5'-TAT HAC RTC BCC YTC YTT ACT GCA-3') using Kapa HiFi HotStart DNA polymerase (Kapa Biosystems, USA), and the PCR protocol as previously noted (see section 3.2.2). The *Bam*HI digested or PCR amplified products were resolved on a 0.7% agarose gel, and ~2kb bands excised and cleaned using a Mega-spin agarose gel extraction kit (Intron Biotechnology, Korea). The *Bam*HI digested products were ligated into a pUC19 vector cut with *Bam*HI, and the PCR products were ligated into pJET1.2 vector (CloneJET™ PCR cloning kit, Fermentas, USA). The plasmid isolated from a single transformed *E. coli* colony was isolated and sequenced by primer walking at Macrogen Inc (Korea).

4.2.3 Bioinformatic analysis

BFDV sequence contigs were assembled into full genome sequences using DNAMAN (version 5.2.9; Lynnon Biosoft). Assembled full genome sequences were aligned with all full BFDV genome sequences available in GenBank, including those isolated from New Zealand and New Caledonia as part of this study (see chapters two and three), using ClustalW (gap open penalty =10; gap extension penalty =5).

Table 4.1. List of species tested for BFDV in Poland.

Scientific name	Common name	Number tested	Number positive
Agapornis sp	Lovebirds	6	0
Alisterus scapularis	Australian King Parrot	4	2
Amazona aestiva	Blue-fronted Amazon	5	1
Amazona amazonica	Orange-winged Amazon	2	1
Amazona barbadensis	Yellow-shouldered Amazon	3	0
Amazona ochrocephala	Yellow-fronted Amazon	2	0
Amazona sp.	Amazon Parrot	2	0
Aprosmictus erythropterus	Red-winged Parrot	4	2
Ara ararauna	Blue and Yellow Macaw	17	0
Ara chloroptera	Red and Green Macaw	8	0
Ara macao	Scarlet Macaw	3	0
Aratinga acuticaudata	Blue-crowned Conure	1	0
Barnardius barnardi	Mallee Ring-necked Parrot	8	0
Barnardius zonarius	Port Lincoln Parrot	2	0
Cacatua alba	White Cockatoo	1	1
Cyanoliseus patagonus	Patagonian Conure	1	0
Diopsittaca nobilis	Red-shouldered Macaw	2	0
Eclectus roratus	Eclectus Parrot	1	0
Elophus roseicapillus	Galah	8	0
Forpus coelestis	Pacific Parrotlet	1	1
Melopsittacus undulatus	Budgerigar	13	9
Pionites melanocephalus	Black-headed Caique	1	0
Platycercus elegans	Crimson Rosella	4	2
Platycercus eximius	Eastern Rosella	2	1
Poicephalus robustus	Cape Parrot	1	1
Poicephalus senegalus	Senegal Parrot	6	2
Probosciger aterrimus	Palm Cockatoo	1	0
Propyrrhura maracana	Blue-winged Macaw	2	0
Psephotus sp.	Grass Parrots	2	0
Psittacula alexandri	Red-breasted Parakeet	3	0
Psittacula cyanocephala	Plum-headed Parakeet	2	0
Psittacula derbiana	Lord Derby's Parakeet	1	0
Psittacula eupatria	Alexandrine Parakeet	3	2
Psittacula krameri	Ring-necked Parakeet	32	9
Psittacus erithacus	African Grey Parrot	52	9
Trichoglossus haematodus	Rainbow Lorikeet	3	0
Total		**209**	**43**

Manual editing of sequence alignments was performed using MEGA5 (Tamura *et al.*, 2011), which was also used to perform pairwise comparisons (p-distance with pairwise deletion of gaps) of the full BFDV genomes, and of the Rep and CP sequences of all available isolates. Maximum likelihood (ML) phylogenies were inferred using PHYML (Guindon *et al.*, 2010), with 1000 non-parametric bootstrap replicates and model GTR+I+G4, as determined by RDP4 (Martin *et al.*, 2010), for the full genome sequences. ML trees of the Rep and CP amino acid sequences were constructed using the LG model in PHYML (Guindon *et al.*, 2010) with aLRT branch support (Anisimova and Gascuel, 2006). Any branches with less than 60% support were collapsed using Mesquite (version 2.75).

Recombination in the Polish BFDV isolates was analysed using RDP4 (Martin *et al.*, 2010) with default setting and the methods RDP (Martin and Rybicki, 2000), GENECONV (Padidam *et al.*, 1999), BOOTSCAN (Martin *et al.*, 2005), MAXCHI (Smith, 1992), CHIMAERA (Posada and Crandall, 2001), SISCAN (Gibbs *et al.*, 2000), and 3SEQ (Boni *et al.*, 2007). Recombination events detected by three or more of these methods with significant p-values, along with phylogenetic support for recombination, were considered to be credible events.

4.3 Results and discussion

4.3.1 PCR screening for BFDV

Of the 209 birds tested, 43 birds (10 genera, 14 species) were positive for BFDV, with positive results found in facilities in 18 different locations across Poland (Fig. 4.1, Table 4.1). There were nine positive results each from the African Grey Parrots (*Psittacus erithacus*; 9/52, 17.3%), Budgerigars (*Melopsittacus undulatus*; 9/13, 69.2%), and Ring-necked Parakeets (*Psittacula krameri*; 9/32, 28.1%). Two each of the Alexandrine Parakeets (*Psittacula eupatria*; 2/3, 66.7%), Australian King Parrots (*Alisterus scapularis*; 2/4, 50%), Crimson Rosellas (*Platycercus elegans*; 2/4, 50%), Red-winged Parrots (*Aprosmictus erythropterus*; 2/4, 50%), and Senegal Parrots (*Poicephalus senegalus*; 2/6, 33.3%) tested positive, along with single specimens of Blue-fronted Amazon (*Amazona aestiva*; 1/5, 20%), Cape Parrot (*Poicephalus robustus*; 1/1, 100%), Eastern Rosella (*Platycercus eximius*; 1/2, 50%), Orange-winged Amazon (*Amazona amazonica*; 1/2, 50%), Pacific Parrotlet (*Forpus coelestis*; 1/1, 100%), and White Cockatoo (*Cacatua alba*; 1/1, 100%).

4.3.2 Full genome analysis of BFDV isolates

Phylogenetic analysis of the full BFDV genomes showed a high degree of divergence, with ten strains represented, based on the system of classification proposed by Varsani *et al.* (2011) (Fig. 4.2). Five new strains were sampled; BFDV-Q (n=4), BFDV-R (n=1), BFDV-S (n=2), BFDV-T (n=10), and BFDV-U (n=1). BFDV-Q had two subtypes, with subtype -Q_1 isolated from two Budgerigars and a Crimson Rosella, and the subtype -Q_2 isolated from a Pacific Parrotlet. BFDV-Q_1 isolates share >98% pairwise identity among themselves, and ~94% identity to Q_2. The ML phylogenetic tree of the full genomes showes BFDV-Q isolates cluster with the budgerigar strains (B-A, -B, -C), with which they share between 85-92% pairwise sequence identity.

BFDV-R_1, isolated from a Blue-fronted Amazon, shares ~92-94% identity with isolates from the BFDV-B strain previously isolated from cockatoos, cockatiels, and galahs in Australia and Japan.

BFDV-S_1 was isolated from two Red-winged Parrots, and appears to share a common ancestor with the BFDV-A strain from New Zealand, with which it shares between ~92-94% pairwise sequence identity, while the two isolates share 98% identity to each other.

BFDV-T isolates formed two subtypes; BFDV-T_1 was isolated from three African Grey Parrots, three Ring-necked Parakeets, two Budgerigars and one Orange-winged Amazon and these isolates share >98.7% identity to each other, and ~96-97% identity to subtype -T_2, isolated from an African Grey Parrot. As a group, the BFDV-T isolates share between 92-94% pairwise sequence identity to BFDV-L and -M isolates from South Africa and Zambia.

Finally, BFDV-U_1 was isolated from an Australian King Parrot, and shares between 92.9-95.7% identity with BFDV-K isolates from Australia, Thailand, Great Britain and the USA.

The remaining 25 isolates were included in existing strains, although new variants of these strains were also detected. A new variant of the existing budgerigar B (B-B) strain, B-B_2, was isolated from Ring-necked Parakeets (n=4), while a new budgerigar strain, B-D_1, was isolated from three budgerigars and one White Cockatoo. This is the first time that these strains have been isolated from species other than the budgerigar. BFDV-B-B_2 isolates share 98.6-99.7% pairwise sequence identity to each other, ~95% identity to BFDV-B-B_1, and between ~91-95% identity to the other budgerigar strains, BFDV-B-A and B-C. BFDV-B-D_1 isolates share >99% identity amongst themselves, and ~94% identity to BFDV-N isolates. However, they share <92% identity with other budgerigar isolates, BFDV-B-A, B-B, and B-C.

Fig. 4.1. Map of Poland, showing locations of BFDV positive samples, species of parrot infected, and virus strain isolated.

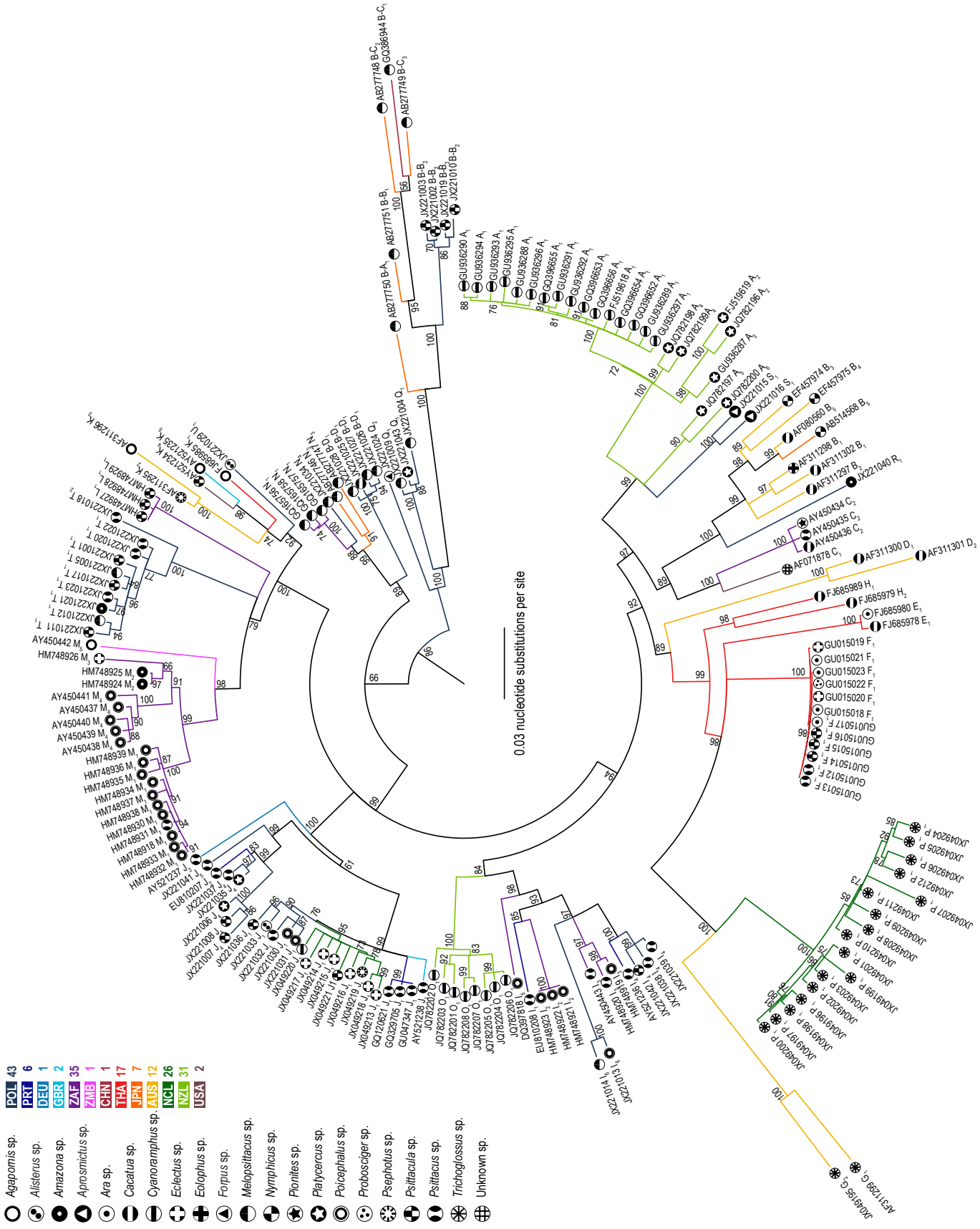


Fig. 4.2. Maximum-likelihood phylogenetic tree showing the relationships of the full genome sequences of the Polish BFDV isolates with all other available full BFDV genome sequences available in GenBank. The tree was constructed in PHYML using model GTR+I+G4, with 1000 non-parametric bootstrap replicates. Colours indicate the country a BFDV sequence was isolated from, with Poland isolates being in dark blue. Symbols indicate the parrot species infected.

The existing strain BFDV-I_4 was isolated from two African Grey Parrots and one Alexandrine Parakeet; these isolates share >99% identity amongst themselves, and ~98% identity to BFDV-I_4 previously isolated from an African Grey Parrot in Portugal. They also share >94% identity with other BFDV-I isolates from Portugal and South Africa. A new subtype, BFDV-I_6, was isolated from a Budgerigar and a Cape Parrot; these isolates share 99.1% identity to each other, ~94% identity to BFDV-I_4 isolated from Poland, and between ~93-95% pairwise sequence identity to other BFDV-I isolates.

The BFDV-J_1 strain was isolated from an African Grey Parrot, an Australian King Parrot, an Alexandrine Parakeet and two Senegal Parrots, and is closely related to BFDV-J_1 isolates previously sampled from Europe and New Caledonia, sharing >97.8% identity with them and >98% identity amongst each other. Two new variants were also isolated: BFDV-J_4, from an African Grey Parrot, a Crimson Rosella, an Eastern Rosella, and two Ring-necked Parakeets, sharing >96% identity amongst themselves; and BFDV-J_5, from an African Grey Parrot. As a group, the BFDV-J strain isolates share >93% identity amongst themselves.

BFDV-N_4, isolated from a Budgerigar, is a new variant of the BFDV-N strain, and shares ~96-99% identity to other BFDV-N isolates. All subtypes of BFDV-N have been isolated from budgerigars.

Of the 18 facilities that had positive results, 11 had infections with only one strain, but in the other seven facilities two or more strains were detected (Fig. 4.1). The presence of multiple strains within one facility increases the chances of birds suffering infections with more than one strain, potentially leading to the evolution of new strains through recombination.

4.3.3 Rep and CP amino acid sequence analysis of BFDV isolates

4.3.3.1 Rep

The Rep sequences of three of the Polish BFDV-B-D_1 isolates share 100% identity with each other, as well as with the Polish BFDV-N_4 isolate and a BFDV-N_2 isolate from Japan, but a fourth BFDV-B-D_1 isolate is slightly more divergent, sharing 99.7% identity with the aforementioned isolates. This is supported by ML phylogenetic analysis of the Rep sequences (Fig 4.3). The BFDV-B-B_2 Rep sequences cluster with a BFDV-B-C_1 isolate from China, and share 97.9-99.7% identity amongst each other and 89.6-90.3% sequence identity with BFDV-B-C_1.

The three BFDV-I_4 Rep sequences share 99-99.3% identity to each other and to an I_4 Rep sequence from a Portuguese isolate, and >98% identity with the I_6 Rep sequences, which are 100% identical.

Polish J_1 Rep sequences share between 97.6-100% pairwise sequence identity with each other, and also with BFDV-U_1, while sharing ~93.4-97.9% identity with the Rep sequences from J_4 isolates, which share between 95.5-99.7% identity amongst themselves. The J_5 Rep sequence shares between 94.4% and 98.3% identity with the J_4 Rep sequences, and ~95-96% identity with J_1 rep sequences, but the ML phylogenetic tree places the J_5 Rep sequence with Rep sequences from A_5 and A_6 isolates from New Zealand, and it shares 99.3% and 100% identity with these respectively.

The BFDV-Q_1 Rep sequences share >99.3% identity amongst themselves, and 95.5-96.2% identity to the Q_2 Rep sequence, which clusters with the BFDV-T strain in the ML phylogenetic tree and shares between 95.5% and 96.2% identity to those Rep sequences. The BFDV-T Rep sequences share between 99.3% and 100% pairwise sequence identity to each other. BFDV-Q_1 clusters with two BFDV-B_1 sequences from Australia and shares between 97.6% and 98.6% identity with them. BFDV-R_1 Rep sequence sits most closely with BFDV-Q, -T, and -L Rep sequences, and shares >94.8% sequence identity with them, while the two BFDV-S_1 isolates share 97.2% identity in the Rep sequence.

4.3.3.2 CP

Maximum likelihood phylogenetic analysis of the CP sequence groups BFDV-B-B_2 with Q_2 (Fig 4.4), and these isolates share between 97.1-97.9% sequence identity, while B-B_2 CP sequences share >99% identity amongst themselves. The B-B_2 strain also shares ~91-95% identity with the B-A_1 and B-B_1 strains. The Q_1 CP sequences share a common ancestor with the BFDV-B-B_1, B-B_2, B-A_1 and Q_2 CP sequences and share 97.5-98.8% identity amongst themselves, ~94-98% identity to B-B_2 CP sequences, and ~90-92% identity with the B-A_1 and B-B_1 CP sequences.

The BFDV-B-D_1 CP sequences are 100% identical to each other, and cluster with strains B-C_1, -C_2, and -C_3, sharing 81.1%, 95.1% and 99.2% pairwise sequence identity with them respectively.

Polish BFDV-I_4 CP sequences share 99.2-99.6% identity amongst themselves, and 96.7-97.6% identity with the CP sequence from a Portuguese BFDV-I_4 isolate, with which they cluster in the ML phylogenetic tree. The Polish -I_4 and -I_6 CP sequences share 91.8-92.7% identity, while the I_6 CP sequences share 99.2% identity. The I_6 CP sequences cluster with I_3 CP sequences, and share 93.4-95.5% identity with them.

Polish -J_1 isolates share 96.7-100% identity amongst themselves, and 94.7-99.6% sequence identity to -J_4 isolates, which share 97.1-100% identity amongst themselves. The -J_5 CP sequences share ~96-99% identity to the Polish -J_1 and -J_4 CP sequences. The Polish BFDV-J CP sequences form a large

group with the CP sequences from all other isolated -J strains from Europe and New Caledonia, suggesting a common origin.

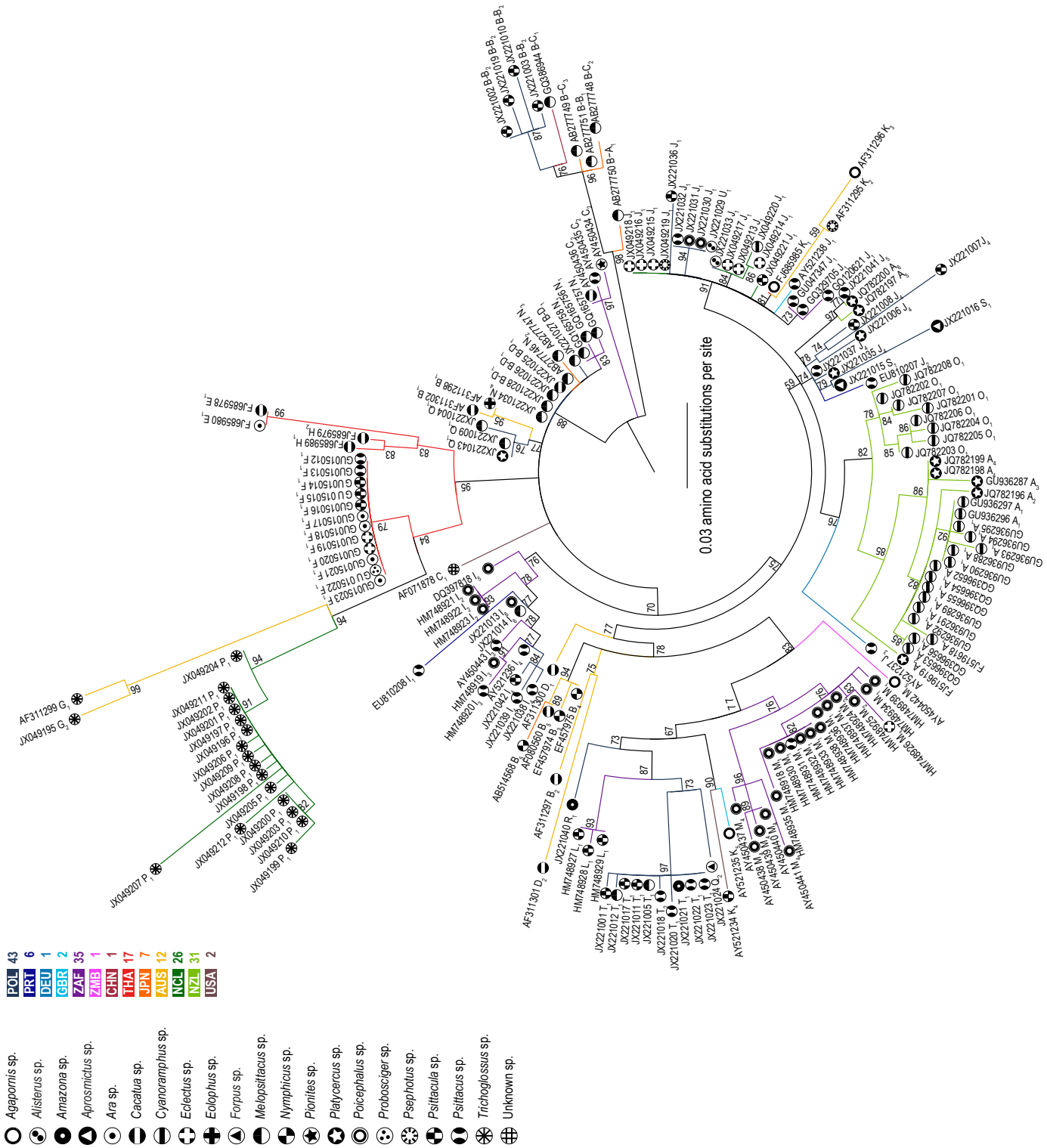


Fig. 4.3. ML phylogenetic tree of the amino acid sequence of Rep from Polish BFDV isolates compared with other Rep sequences available in GenBank. The tree was constructed in PHYML using the LG model with aLRT branch support. Polish isolates are in dark blue.

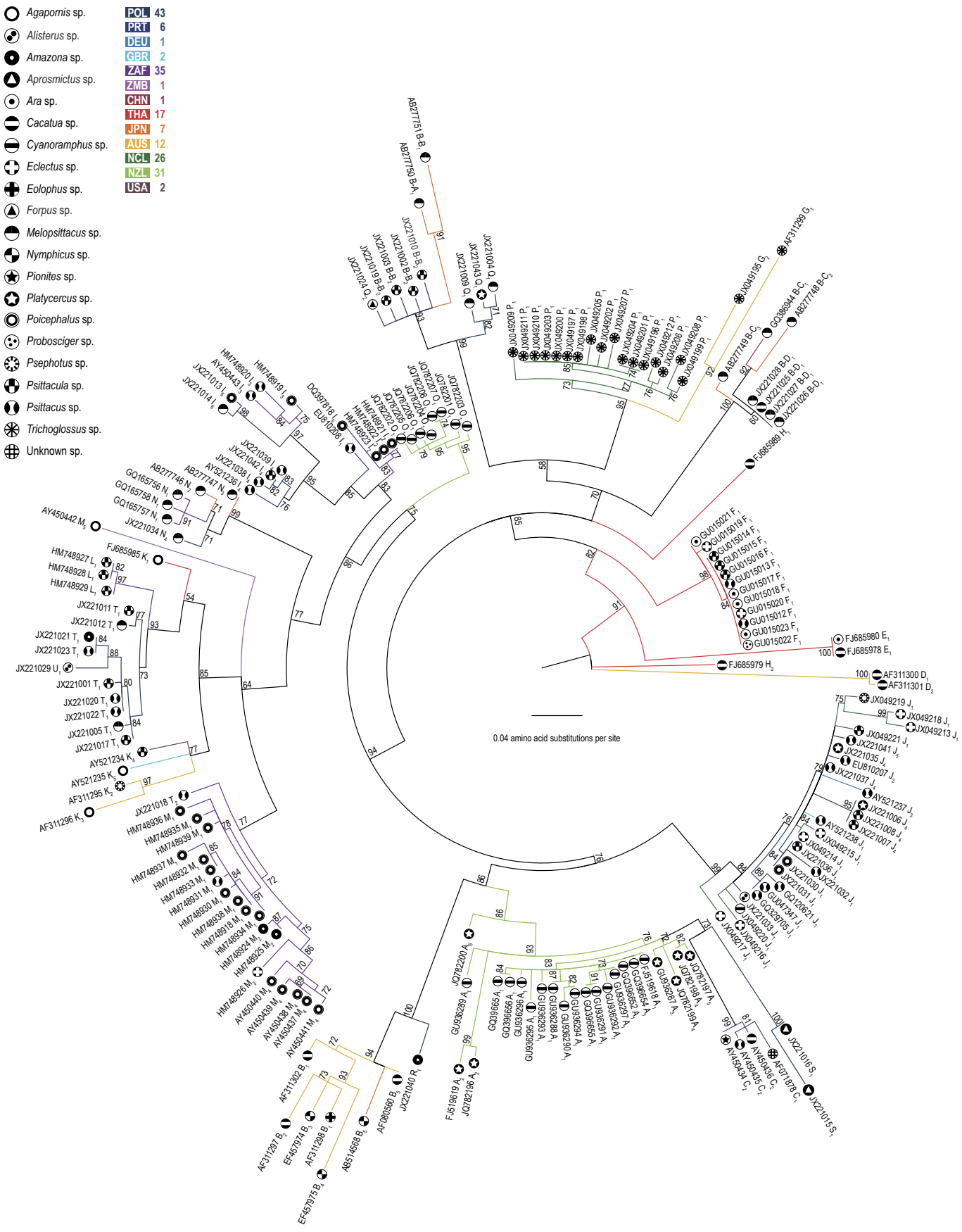


Fig. 4.4. ML phylogenetic tree, constructed in PHYML using the LG model with aLRT branch support, of the relationships of the CP amino acid sequences from BFDV isolates. Polish isolates are in dark blue.

Maximum likelihood phylogenetic analysis places the CP sequence from BFDV-N_4 with those from BFDV-N_1, N_2, and N_3, previously isolated from South Africa and Japan, and the -N_4 CP sequence shares 96.3-98% identity with these CP sequences. The CP sequence of the BFDV-R_1 strain groups with CP sequences from BFDV-B strains isolated in Australia and Japan, and shares between 89.7-94.7% identity with them.

BFDV-S_1 CP sequences have 95.9% identity to each other, and share 82.4-88.1% identity with CP sequences from BFDV-C strains from South Africa and the USA. They share higher identity with CP sequences from BFDV-A strains (83.6-92.2%), with which they form a larger cluster.

CP sequences from the two BFDV-T subtypes, -T_1 and -T_2, cluster separately in the phylogenetic tree, and share 93.4-94.3% identity. BFDV-T_1 CP sequences form a cluster with BFDV-U_1, with which they share between 96.3% and 97.1% identity, while -T_1 CP sequences share 96.7-100% identity amongst themselves. The -T_2 subtype clusters with BFDV-M CP sequences from South Africa, sharing 94.3% to 97.1% identity with them.

4.3.4 Recombination analysis

Evidence of recombination was detected in a number of Polish BFDV isolates (Fig. 4.5). BFDV-B-B_2, isolated from four Ring-necked Parakeets, was the most highly recombinant strain, with five separate recombination events detected. The first event, a, in the 5' intergenic region and the N-terminal portion of the *rep* gene, is an ~600 bp fragment from a BFDV-B-A_1 strain from Japan. Within this recombinant region is a second ~285bp fragment of unknown origin. Events c and d overlap within the *rep* gene, with c being an ~250bp fragment from a Japanese BFDV-B-C_2 strain, and d being an ~370bp fragment from BFDV-B-C_1 from China. The fifth recombination event, e, was also detected in a single BFDV-Q_1 isolate from a budgerigar, and is an ~300 bp fragment from a South African BFDV-N_1 strain. The recombination region spans the C-terminal portion of the *rep* gene and the 3' intergenic region. The four BFDV-B-D_1 isolates are all recombinants, with a fragment of either ~950bp or ~780bp from the BFDV-P_1 strain from New Caledonia (event f). The BFDV-J_1 isolates are all recombinants, with an ~800bp fragment from a BFDV-J_5 isolate from Poland (event g). A recombination event was detected both in a single BFDV-J_4 isolate from a Ring-necked Parakeet, with an ~120bp fragment within the *rep* from an unknown BFDV variant (event h), and in a single BFDV-J_5 isolate from an African Grey Parrot, with an ~330bp fragment in the C-terminal portion of the *cp* and the intergenic region, from a Polish BFDV-U strain (event i). The other two BFDV-Q_1 isolates have an ~360bp recombinant region spanning the C-terminal portion of rep and the intergenic region, from a Japanese BFDV-N_2 isolate (event j), while the single BFDV-Q_2 isolate has a large ~980bp fragment from a Polish BFDV-Q_1 isolate, spanning the *cp* gene and extending into the

5’ intergenic region (event k). The single BFDV-R_1 isolate from a Blue-fronted Amazon has a fragment of ~770bp from an Australian BFDV-B_5 strain, in the N-terminal portion of the *cp* gene and the 5’intergenic region (event l). Both the BFDV-S_1 isolates were recombinants, with an ~620bp fragment in the C-terminal portion of the *rep* gene, from a Polish BFDV-J_5 strain (event m). BFDV-T_2, isolated from an African Grey Parrot, has a recombinant region in the N-terminal portion of the *cp* gene and the 5’ intergenic region, with the ~640bp fragment being of unknown origin (event n), and the BFDV-U_1 isolate from an Australian King Parrot has a similar recombinant region with a similar sized fragment from a BFDV-T_1 strain from Poland (event o).

This level of recombination within Polish strains, along with the large number of strains sampled, is unsurprising when considering the long history Europe has had with importing exotic parrots. The captive facilities that these birds are generally contained in provide the ideal environment for evolution of BFDV through recombination, and the six previously unsampled strains, BFDV-Q, -R, -S, -T, -U, and BFDV-B-D are evidence of the emergence of new strains through recombination. That there were recombinant sequences of unknown origin suggests that there are even more strains yet to be sampled.

Event	Recombinant(s)	Recombinant region	Potential Major Parent	Potential Minor Parent	Detection Method	P-value
a	JX221019-B-B_2, JX221002-B-B_2, JX221003-B-B_2, JX221010-B-B_2	1826-448	Unknown	AB277750-B-A_1	RGBMCST	$2.95x10^{-31}$
b	JX221019-B-B_2, JX221002-B-B_2, JX221003-B-B_2, JX221010-B-B_2	62-347	JX221024-Q_2	Unknown	RGMCST	$4.48x10^{-15}$
c	JX221019-B-B_2, JX221002-B-B_2, JX221003-B-B_2, JX221010-B-B_2	475-730	JX221043-Q_1	AB277748-B-C_2	RGBMCST	$7.90x10^{-14}$
d	JX221019-B-B_2, JX221002-B-B_2, JX221003-B-B_2, JX221010-B-B_2	468-840	HM748921-I_2	GQ386944-B-C_1	GBMCT	$1.02x10^{-11}$
e	JX221019-B-B_2, JX221002-B-B_2, JX221003-B-B_2, JX221010-B-B_2, JX221009-Q_1	919-1232	Unknown	GQ165758-N_1	RGBMCST	$1.59x10^{-13}$
f	JX221027-B-D_1, JX221028-B-D_1,	1069-27	AB277746-N_2	JX049199-P_1	GMC	$9.37x10^{-9}$
	JX221025-B-D_1, JX221026-B-D_1	1085-1866				
g	JX221030-J_1, JX221036-J_1, JX221033-J_1, JX221032-J_1, JX221031-J_1	1162-1988	JX221029-U_1	JX221041-J_5	RGMCST	$5.91x10^{-29}$
h	JX221007-J_4	420-540	JX221008-J_4	Unknown	RGBT	$1.89x10^{-6}$
i	JX221041-J_5	1055-1388	Unknown	JX221029-U_1	RGBMST	$9.56x10^{-10}$

Fig 4.5. Cartoon illustration and details of recombination events detected in Polish BFDV isolates using RDP4 and the methods RDP (R), GENECONV (G), BOOTSCAN (B), MAXCHI (M), CHIMAERA (C), SISCAN (S) and 3SEQ (T). Only detection methods with significant results are shown. The p-value is for the detection method shown in bold. (Figure continued next page).

j	JX221043-Q_1, JX221004-Q_1	874-1234	Unknown	AB277746-N_2	RGB**M**CT	6.21x10^{-11}
k	JX221024-Q_2	1077-56	JX221012-T_1	JX221004-Q_1	RGB**M**CT	3.22x10^{-15}
l	JX221040-R_1	1445-223	AF311296-K_3	AF080560-B_5	RGB**M**CST	4.48x10^{-12}
m	JX221015-S_1, JX221016-S_1	431-1055	JQ782198-A_4	JX221041-J_5	RGMCST	5.82x10^{-26}
n	JX221018-T_2	1412-56	PL-410-T_1	Unknown	GMC	1.08x10^{-8}
o	JX221029-U_1	1360-2002	FJ685985-K_1	JX221023-T_1	RGMCT	7.96x10^{-7}

Fig. 4.5. continued

4.4 Concluding comments

The results from this study indicate that there is a high degree of genetic diversity among BFDV isolates in Poland, indicating that there have probably been multiple introductions of the virus over an extended period of time. Given the widespread trade in parrots between countries within European borders, it could be expected that there is a similar high degree of diversity in BFDV isolates throughout the continent. Further, we cannot ignore the fact that there is continual 'seeding' of BDFV strains from infected breeding stock acquired from outside Europe. Phylogenetic analysis of the full genomes assigned the BFDV isolates to ten different strains, over half of which have not been sampled previously (BFDV-Q, -R, -S, -T, and -U, and BFDV-B-D), and were isolated from approximately half of the BFDV-positive birds (n=22). Of the four previously sampled strains (BFDV-B-B, BFDV-I, -J, and -N), only eight isolates were from existing subtypes (BFDV-I_4 and BFDV-J_1), with the remainder of the isolates (n=13) representing new subtypes of existing strains (BFDV-B-B_2, BFDV-I_6, BFDV-J_4, -J_5, and BFDV-N_4).

For the first time, previously assumed budgerigar specific strains were found to be infecting other species. BFDV-B-B_2, a new subtype, was isolated from four Ring-necked Parakeets, while a new strain, BFDV-B-D_1 was isolated from three Budgerigars and a White Cockatoo. However these isolates were subsequently shown to be recombinant, and with five separate recombination events BFDV-B-B_2 in particular was highly recombinant. Analysis showed that budgerigar specific strains potentially contributed to the recombination events in BFDV-B-B_2 isolates (BFDV-B-A_1, event a; BFDV-B-C_2, event c; BFDV-B-C_3, event d; BFDV-N_1, event e; Fig. 4.5). Dual infections within budgerigars may have allowed recombination to occur, leading to the emergence of these new strains with altered pathogenicity. The BFDV-N strain, however, has still only been recovered from budgerigars, although recombination with this strain may have contributed to the emergence of strain -Q, found in two Budgerigars, a Crimson Rosella and a Pacific Parrotlet (Fig. 4.5). The fact that unknown sequences were detected in recombination events indicates that the diversity within Poland, and accordingly, likely in much of Europe, is much greater than what has been revealed here.

While Europe has no native parrot species, and thus there is no real concern that the spread of the virus to the wild is going to place undue threat on any birds, endangered exotic parrots are often housed within captive facilities for breeding to boost wild populations, or to raise public awareness about the plight of endangered species. BFDV poses a risk to these birds, especially given the fact that transmission of the virus is enhanced in captive environments. Europe also exports parrots bred within captive facilities, thus contributing to the global spread of BFDV, and placing other

endangered parrots at increased risk. The long-term fascination that Europe has had with exotic parrot species may have resulted in the continent becoming a major centre for evolution of BFDV through recombination of different strains.

Chapter five: Summary and Concluding Comments

5.1 BFDV summary

5.1.1 Virus characteristics

Psittacine beak and feather disease (PBFD) is a potentially fatal disease of parrots, caused by *Beak and feather disease virus*, a non-enveloped, icosahedral, ssDNA virus of the *Circoviridae* family. Since the first observations and description of PBFD in Australia in the 1970's and 80's (Pass and Perry, 1984) the disease has been reported on every continent, with the exception of Antarctica. The global spread of the virus has been facilitated by the international trade in exotic parrots, which occurs both through regulated legal channels, but also through illegal trafficking, often of wild caught endangered species. Circoviruses have been reported to be very stable and resistant to many methods of inactivation (Urlings *et al.*, 1993; Welch *et al.*, 2006; Yuasa, 1992), increasing the possibility of transmission of infection through environmental contamination. Attempts to develop a vaccine to protect against infection have been hampered due to the inability to culture BFDV *in vitro*, and there is currently no effective treatment or cure available, simply palliative care for chronically infected birds (Raidal *et al.*, 1993b; Ritchie *et al.*, 1992). Further, there has been limited success in making recombinant BFDV vaccines. (Bonne *et al.*, 2009). With over a quarter of all extant psittacine species considered at risk of extinction in the wild, the widespread presence of such an endurable and highly infectious virus as BFDV poses an additional challenge for conservation management strategies aimed at increasing the populations of such endangered birds.

5.1.2 Genetic diversity

Initial studies into BFDV indicated little difference between isolates, leading to the assumption that only one strain existed that all psittacine species were susceptible to (Bassami *et al.*, 1998; Niagro *et al.*, 1998; Ritchie *et al.*, 1990). However reports of differences in symptom presentation and disease progression between species led to investigations into the possible existence of different strains, and whether any sort of species or geographical specificity could be attributed to them (de Kloet and de Kloet, 2004; Kock *et al.*, 1993; Ritchie *et al.*, 2003). Full genome analyses of BFDV isolates have

indicated that different strains do exist, which may originally have exhibited a degree of host and/or regional specificity, but the international trade in exotic psittacines and the practice of combining multiple species in captive facilities has enabled evolution via recombination, resulting in the emergence of new strains with altered pathogenicity, effectively blurring the lines of specificity (Varsani *et al.*, 2011). In no place is this more evident than in Europe, which has a long history of importing exotic parrots, and in this study has exhibited the greatest degree of genetic diversity between isolates along with the highest recombination rates (Chapter four, Fig. 4.5).

5.1.3 Preventative measures

In order to prevent the spread of BFDV, certain measures need to be taken. All breeding facilities should undertake regular screening of their birds to ensure they are virus free, and any birds that are brought in should be tested and quarantined before joining the established population. Outdoor aviaries should be covered, so that any wild parrots that may be infected cannot contaminate the aviary with feather dander or faecal matter, which may result in captive birds being infected. These prevention strategies also hold true on a larger scale. Any parrots destined for the pet trade need to be screened before they leave their country of origin, and when they arrive at their destination, need to undergo a quarantine period, along with additional screening, to determine their BFDV status. Only if repeatedly negative tests are obtained during the quarantine period should they be allowed to join an existing aviary, which should also have a BFDV negative status for all birds.

Implementation of similar protocols would be an advantageous addition to conservation management strategies in place for dealing with the recovery of endangered psittacine species. Currently, many such strategies include rearing birds in captivity for release to the wild, or translocation of individuals either between populations to increase genetic diversity, or to predator free sanctuaries such as offshore islands in the hopes that the population can recover in the absence of threat by introduced mammalian predators. However, without comprehensive testing regimes these methods can result either in the introduction of BFDV to a BFDV-naïve population, or BFDV-naïve birds into an environment where BFDV is present. While the virus itself may not kill the birds, the reduction in immune system function caused by BFDV infection can leave the bird open to secondary infections, which can have a fatal outcome.

Adherence to such stringent screening protocols can minimise the spread of BFDV into areas where it may not yet be present, and reduce the risk of introducing different strains into areas where BFDV is present, and thus prevent recombination between different strains which can lead to the emergence of novel strains.

5.2 Global status of BFDV

5.2.1 Origin and distribution of BFDV, and the threat imposed on endangered parrots

With the first described cases of PBFD occurring in Australia, it is generally widely assumed that BFDV has its origins there, but there is currently no incontrovertible evidence to prove this. On the contrary, full genome analysis of BFDV isolates suggests otherwise, as the basal group of isolates are isolated from budgerigars from Japan and China (Fig. 4.2). While BFDV is considered endemic among Australian wild parrots, it is now present worldwide in both wild and captive populations. The role that the international trade in exotic parrots has played in spreading BFDV has been demonstrated in Europe, which has obviously had multiple introductions of the virus during its long history with parrot importation. Europe also exports parrots bred in captivity, which has contributed to the spread of BFDV. The infection of captive birds, including a vulnerable native parakeet, with the BFDV-J_1 strain in New Caledonia is clearly attributable to the introduction of an infected parrot from Europe. BFDV was also potentially introduced to New Zealand along with the Eastern Rosella in the 1900's, which has resulted in the vulnerable Red-fronted Parakeet becoming infected.

Any at risk psittacine species are obviously affected to a greater degree by the global spread of BFDV, as it imposes an additional threat to survival. A notable example is that of the Echo Parakeet (*Psittacula echo*), native to the island of Mauritius. Although an intensive management program has seen the population of this endangered species increase to ~500 individuals today, this progress suffered a setback when an outbreak of BFDV during the 2005/2006 breeding season resulted in the death of some birds, with some having to be euthanized (Kundu *et al.*, 2012). The presence of BFDV on the island is thought to be due to introduced psittacine species such as the Ring-necked Parakeet (*Psittacula krameri*). While the population did recover, this outbreak also forced the adoption of management techniques that were more hands-off, as human activity was implicated in contributing to the spread of the virus (Kundu *et al.*, 2012). The South African Cape Parrot (*Poicephalus robustus*) has also been shown to be susceptible to BFDV (Heath *et al.*, 2004), and it is possible that the disease has contributed to the decline of this endangered parrot in the wild (Wirminghaus *et al.*, 1999).

Given the potentially devastating effects an outbreak of BFDV could have on a naïve population, the case of individuals of the critically endangered New Zealand Kakapo being translocated from Codfish Island to Little Barrier Island, where BFDV has been found among the Red-fronted Parakeet population, will be one to watch. While it is important to determine whether the Kakapo can survive and breed on their own without the intensive human management currently in place on Codfish

Island, it must be questioned whether transferring them to an environment where they could be facing an additional threat to survival is the right choice. Equally, the transfer is essential to determine whether Kakapos are resistant to BFDV as this has conservation implications in terms of identifying new locations for their breeding programs.

5.2.2 The importance of full genome analysis.

The first BFDV genomes were fully sequenced in 1998 (Bassami *et al.*, 1998; Niagro *et al.*, 1998), a full 14 years after the first clinical description of the disease indicated that a virus was the most likely causative agent (Pass and Perry, 1984). While this allowed the development of primers enabling PCR to be used as a rapid diagnostic tool for BFDV infections (Ritchie *et al.*, 2003; Ypelaar *et al.*, 1999), there has since been limited interest in isolating and sequencing full viral genomes for downstream bioinformatic applications. In most cases, studies into the evolutionary history of BFDV have focused on sequencing merely a portion of one of the major ORF's, Rep or CP. However these genes evolve at different rates, with the Rep gene tending to be more conserved and the CP gene more variable (Bassami *et al.*, 2001; Heath *et al.*, 2004; Raue *et al.*, 2004; Varsani *et al.*, 2011), and ssDNA viruses have been shown to be highly recombinant (Lefeuvre *et al.*, 2009), so any phylogenetic analysis focusing purely on one of these genes is unlikely to provide a comprehensive and accurate assessment of phylogenetic relationships between isolates. A selection analysis of all full BFDV genomes available in GenBank shows that the Rep, with 0.62 substitutions per site, has seven codons under positive selection and 132 under negative selection, while CP has 0.88 substitutions per site, six codons under positive selection, and 108 under negative selection. A recombination analysis should be performed as part of any full genome analysis, to determine how it is affecting evolution. Recombination is common among ssDNA viruses, and allows a rapid way to explore the available sequence space, whereby different combinations of entire sections of genomes from different isolates can be sampled. This enables evolution to occur much more rapidly than through mutation alone, resulting in the rapid emergence of new strains. The budgerigar strains B-A, -B, and -C are all highly recombinant, being almost entirely made up of components derived from other strains. This high degree of recombination may have contributed to their being isolated from species other than the budgerigar, when they were considered different enough at the nucleotide level that it was suggested they be classed as a new budgerigar circovirus species (Varsani *et al.*, 2011). In contrast, the BFDV-N strain, which has still only been isolated from budgerigars, shows no signs of any recombination events within any isolates.

The maximum likelihood phylogenetic tree based on the full genome sequences of all BFDV isolates currently available in GenBank reveals some interesting things (Fig. 4.2). Some strains have only ever

been isolated from one country, such as BFDV-A from New Zealand, or BFDV-P from New Caledonia, while others have been isolated from different countries, such as BFDV-J, which appears to have had its origins in Europe, being isolated from all four countries sampled, before spreading to New Caledonia. BFDV-B has been isolated only from members of the *Cacatuidae* family, while a common ancestor appears to have led to two strains, BFDV-P in New Caledonia, and BFDV-G in Australia, which are divergent from other strains and have been found only in *Trichoglossus* species, lending credence to the existence of a lorikeet specific lineage. Analysis of full genome sequences can also reveal evidence of outbreaks, as seen in the BFDV-F1 strain from Thailand, which provides a classic example of an infection rapidly spreading throughout a captive facility in which multiple species are housed.

The use of ϕ29 DNA polymerase has made it possible to amplify entire circular ssDNA genomes literally overnight, which can then be readily cloned and sequenced using widely available and relatively cheap molecular techniques. Using these techniques, the studies described in this thesis have contributed to the addition of 78 full BFDV genome sequences to GenBank in a little over a year (eight isolates from Yellow-crowned Parakeets from the New Zealand study; 26 genomes from New Caledonia; 43 genomes from Poland). If we are to obtain the full picture of how BFDV has evolved since its emergence and is still evolving, and wish to track the origin of the virus, it can only be done through analysis of full viral genomes.

5.3 Future directions

Including the 78 full genomes deposited as part of this study, the number of full BFDV genome sequences currently available in GenBank stands at just 184, a relatively low number given the global distribution of BFDV. In particular, there is a surprising lack of full sequences available from Australia or the Americas. There have been 52 full genome sequences deposited from Europe, 36 from Africa, and 25 from Asia. If Australasia is considered as a whole, there have been 69 full genomes sequenced, but only 12 of these have come from Australia, the largest landmass with the greatest parrot diversity, and one of the 12 was sequenced as part of this study. From the USA, only two full BFDV genomes have been sequenced, one of which was derived from pooled blood so the infected psittacine species cannot be determined. There are no sequences in GenBank that have originated from South America, although some have been isolated from South American species such as *Amazona* and *Ara* species. This is hardly indicative of a lack of infection in these countries; in Australia at least, BFDV is considered the most common viral disease among wild parrot populations

(Raidal *et al.*, 1993c). Research groups from both countries have also been instrumental in conducting a great deal of the early research into characteristics of both PBFD and BFDV, so opportunities have certainly existed to isolate and sequence full BFDV genomes. If any samples from these early studies have been archived, it may still be possible to go back and isolate full viral genomes from them.

While a greater sampling effort in Australia and America would be beneficial to get a more accurate picture of the true global diversity of isolates, continued global testing is also indicated, of as many different psittacine species as possible, from as many different regions as possible. Given the centuries of popularity parrot species have enjoyed in Europe, it could be expected that the diversity among isolates in European countries other than Poland will be equally high, and there are certainly more strains that have yet to be sampled, possibly among the wild populations that have become established, but certainly among captive populations. Testing of wild native parrots in New Caledonia could provide an indication of whether or not BFDV has begun to affect them, and thus provide crucial information needed to make appropriate conservation management decisions. The situation is similar in New Zealand; continued screening of wild native parrots can provide information on how fast the virus is spreading among already infected populations, and, in particular with the Kakapo, allow for early detection of infection so that appropriate measures can be taken. The more that is known about BFDV, the better equipped we are to develop conservation plans that can protect the most vulnerable parrot species, or potentially develop an effective treatment or even a cure for infected birds. Any available archived samples could provide interesting comparisons between isolates present within a region or species today, and those present 10-20 years ago. Ultimately, the larger the dataset of full BFDV genome sequences is, the easier it will be to establish phylogenetic relationships with a greater degree of confidence. Eventually, with a large enough dataset, it may be possible to determine the true origin of infection.

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